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Complications from metastatic disease are the primary cause of death in breast cancer. We found that human breast cancer cells can express the adhesion receptor integrin ạvḅ3 in an activated or a non-activated functional form. Expression of the activated, but not the non-activated receptor supports metastatic dissemination. Mechanisms through which activated integriṇ avḅ3 promotes metastasis include: binding of soluble ligand, support of tumor cell arrest during blood flow through tumor cell interaction with platelets, and promotion of breast cancer cell migration. Activated integrin ạvḅ3 promotes breast cancer migration by cooperating with metalloproteinase MMP-9. A model mechanism for this cooperation is proposed. Our new findings support the originally proposed concept, that activation of breast cancer cell integriṇ avḅ3 supports the metastatic phenotype in the tumor cells. Thus, these studies identify activateḍ avḅ3 as a new functional marker of metastatic breast cancer cells and indicate that activateḍ avḅ3 should be considered as a novel target for the inhibition of breast cancer metastasis.

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INTRODUCTION:

Complications from *metastatic disease* are the primary cause of death in breast cancer. The *purpose* of this project is to analyze adhesive mechanisms in breast cancer metastasis, because these may critically determine breast cancer progression and may represent a target to combat the disease. We found that human breast cancer cells can express the adhesion receptor, integrin αvβ3 in an activated or a non-activated state. We *hypothesized that breast cancer cell integrin αvβ3 activation determines the metastatic phenotype of the tumor cells. Our results validate this hypothesis and indicate a causal relationship between breast cancer cell integrin αvβ3activation and an increase in metastatic activity.* During the past funding period we made key observations, which indicate that expression of integrin αvβ3 in a functionally activated state endows breast cancer cells with the ability to attach under physiological blood flow conditions as in the vasculature, and with the ability to migrate toward specific matrix proteins. Our results further indicate that activated integrin αvβ3 acts in concert with metalloproteinase MMP-9, and together these molecules promote a migratory and metastatic phenotype in breast cancer cells. Thus, our latest findings consolidate our working hypothesis and provide information toward a mechanism, through which expression of activated integrin αvβ3 promotes specific adhesive functions that determine the metastatic potential in breast cancer cells.

BODY:

Integrin αvβ3 has been implicated in the pathophysiology of malignant tumors [1]. It clearly has a role on endothelial cells where it is required for tumor angiogenesis [2]. In several malignancies, however, αvβ3 was also found on the tumor cells, and expression correlates with tumor progression in melanoma, glioma, ovarian and breast cancer [3-7]. In breast cancer, αvβ3 expression characterizes the metastatic phenotype, as this integrin is clearly upregulated in invasive tumors, particularly in bone metastases [8]. A mechanistic role of αvβ3 in the spread of breast cancer, however, has yet to be established. We previously suggested that an interaction of circulating tumor cells with platelets represents a potential mechanism for tumor cell arrest within the vasculature [9]. During the first year of the funding period, we showed that integrin αvβ3 supports breast cancer cell attachment under blood flow conditions in an activation dependent manner. Integrin αvβ3 was found in tow distinct functional states in human breast cancer cells. The activated, but not the non-activated state supports tumor cell arrest during blood flow through interaction with platelets [10]. Importantly, activated αvβ3 is expressed by freshly isolated metastatic human breast cancer cells and variants of the MDA-MB 435 human breast cancer cell line, derived from mammary fat pad tumors or distant metastases in severe combined immune deficient mice (SCID) [10] Expression of constitutively activated mutant $\alpha v\beta 3_{D723R}$, but not $\alpha v\beta 3_{WT}$, in MDA-MB 435 cells strongly promotes metastasis in the mouse model [10]. Thus, breast cancer cells can express a platelet-interactive and metastatic phenotype that is controlled by the activation of integrin αvβ3. Breast cancer is almost always associated with occult or overt thromboembolic events, and malignancy is generally recognized as an acquired thrombophilic condition [11]. Our studies provide a mechanism through which a specific adhesive interaction between tumor cells and platelets can support breast cancer metastasis [12]. Consequently, alterations within tumors that lead to the aberrant control of integrin activation are expected to adversely affect the course of human breast cancer (*Please see appended publications).*
The following information has not been published yet. Manuscripts are in preparation.
For the second year of the funding period, we report five major findings/accomplishments: 1. Primary

metastatic tumor cells from malignant effusions and peripheral blood samples of patients with stage IV breast cancer express integrin αvβ3 in its activated functional state; 2. A primary metastatic cell line established from a blood sample of a patient with metastatic breast cancer, serves as a new cell model for studies of human breast cancer in immune deficient mice; 3. Activated integrin αvβ3 promotes and enhances breast cancer cell migration toward extracellular matrix proteins; 4. Activated αvβ3 cooperates with metalloproteinase MMP-9 during support of breast cancer cell migration; and 5. In our analyses of the modulation of integrin αvβ3 function during metastatic progression, we found mutations in the integrin β3 subunit gene of highly metastatic variants of our MDA-MB 435 breast cancer cell model. The mutations are located in regions of the molecule that are important for β3 integrin ligand binding functions.

1. Primary metastatic tumor cells from malignant effusions and peripheral blood samples of patients with stage IV breast cancer express integrin αvβ3 in its activated functional state.

To test a clinical relevance of the expression and function of activated integrin αvβ3 in breast cancer metastasis, we isolated circulating tumor cells from patients with stage IV breast cancer. Circulating metastatic cells were isolated from peripheral blood samples of breast cancer patients using immuno magnetic beads, decorated with a monoclonal antibody directed against a human epithelial antigen (mab Ber EP-4) (Fig. 1). Processing of about 25 blood samples resulted in the establishment of three primary metastatic human breast cancer cell lines from circulating tumor cells. These cell lines were termed BCM-1, BCM-2 and BMS. The freshly isolated cells were analyzed for integrin expression and function, as soon as sufficient cell numbers were obtained (passage 2-3). Each of the cell lines expressed integrin αvβ3, and the receptor was found to be in a constitutively activated form based on functional analyses: The cells were able to bind soluble ligands in an αvβ3 dependent manner, and arrested during blood flow through αvβ3 mediated interaction with platelets (Fig. 2). Additional functional criteria, that identified αvβ3 as constitutively activated in these primary metastatic breast cancer cell lines, are the ability of the receptor to support enhanced binding to stimulated endothelial cells during blood flow, and to mediate fibrinogen directed migration (see below).

2. A primary metastatic cell line established from a blood sample of a patient with metastatic breast cancer serves as a new cell model for studies of human breast cancer in immune deficient mice.

Amongst the three newly established cell lines, BCM-2 shows the most aggressive phenotype, as judged by the specific adhesive cell functions that are mediated by activated integrin αvβ3. Therefore, we tested the metastatic activity of BCM-2 cells in immune deficient mice. In a preliminary study, we found that this cell line is a useful new model for the analysis of human breast cancer metastasis in SCID mice (Fig. 3). When injected into the lateral tail veins of 8 week old female SCID mice, BCM-2 cells rapidly established metastases in the lungs. Metastatic foci were readily detectable at the surface of the lungs six weeks after tumor cell inoculation. In the coming funding period, we plan to analyze the kinetics of BCM-2 metastasis and target organ specificity. Thus, we plan to include examination of other major target organs of breast cancer metastasis, such as bone, liver and brain. We hope to establish a rapid detection method to analyze and quantify the metastatic colonization of target organs very early after tumor cell inoculation. Metastatic burden will be examined based on detection of micrometastases by quantitative PCR, targeting human specific sequences, such as the Alu repeats [13,14]. The other primary

metastatic human breast cancer cells are currently under investigation in the SCID mouse model. Our preliminary *in vivo* data indicate that BCM-2 is a useful new human breast cancer cell model that will allow us to address the role of specific adhesive breast cancer cell functions during metastatic dissemination in immune deficient mice. This new cell model is a much needed expansion of the very limited number of currently available cell models for human breast cancer metastasis.

Together, these data indicate a clinical relevance of the expression of integrin αvβ3 in an activated functional state in primary metastatic cells from breast cancer patients. The activated receptor endows the tumor cells with specific adhesive functions, which are likely to promote the metastatic phenotype.

3. Activated integrin αvβ3 promotes and enhances breast cancer cell migration toward extracellular matrix proteins.

The above data define integrin αvβ3 in its activated form as a functional marker of metastatic human breast cancer cells. A key functional characteristic of activated αvβ3 is its ability to support tumor cell arrest during blood flow, based on tumor cell binding to adherent activated platelets [10]. The profound effect of the activation state of breast cancer cell integrin αvβ3 on the metastatic activity of the tumor cells prompted us to analyze, which other adhesive or ligand binding functions in tumor cells are determined by activation state of αvβ3. Recent findings in the literature indicate that integrin activation can critically affect cell migration [15]. Therefore, we compared variants of the MDA-MB 435 human breast cancer cell line, which express integrin αvβ3 either in an activated or a non-activated state, for their abilities to migrate toward the matrix proteins vitronectin, fibronectin, von Willebrand Factor and fibrinogen (Fig. 4). These matrix proteins are components of the basal lamina and subendothelial matrix. Therefore, these proteins represent relevant substrates that are likely encountered by metastasizing breast cancer cells. Fibrinogen deserves special attention, because it is a prominent constituent at multiple sites that are important during breast cancer metastasis[16]. These include the primary tumor, sites of tumor cell entry into the vasculature and sites of tumor cell extravasation. As an initial step during metastatic dissemination, tumor cells secrete a vascular permeability factor that renders the local microvasculature hyperpermeable to fibrinogen and to other plasma proteins. Therefore, breast cancer cell interaction with a fibrinogen or fibrin substrate may be critical during metastatic dissemination.

To analyze a clinical relevance of a role for activated integrin αvβ3 in breast cancer cell migration, we included freshly isolated metastatic cells from peripheral blood samples of stage IV breast cancer patients (Fig. 4). Integrin αvβ3 was defined activated when it bound soluble ligand and supported tumor cell arrest during blood flow through interaction with platelets. Based on these criteria, αvβ3 is non-activated in parental MDA-MB 435 cells and in a β3-wild type transfected β3-minus variant of the parental cells. αvβ3 is activated in $\beta3_{D723R}$ transfected β3-minus cells (constitutively activated β3 mutant) and in *in vivo* selected MDA-MB 435 variants from metastases in SCID mice, as well as in primary metastatic cells from breast cancer patients. In these cell types, activated αvβ3 enhanced breast cancer cell migration toward vitronectin and fibronectin, and was required for migration toward fibrinogen (Fig. 4, 5). In breast cancer cells expressing non-activated αvβ3 (MDA-MB 435 parental cells), fibrinogen directed migration could not be promoted by experimental integrin activation with Mn^{2+} (Fig. 6), but by a soluble factor produced by breast cancer cell variants that express activated αvβ3 (Fig. 7). Fibrinogen

directed migration of breast cancer cells, which *per se* express non-activated αvβ3, induced by the soluble factor, was mediated by αvβ3 (Fig. 8). Promotion of breast cancer cell migration by the soluble factor was specific for fibrinogen as a haptotactic migration substrate. Migration toward vitronectin and fibronectin was not affected (Fig. 9). From these results, we conclude that integrin αvβ3 is required for breast cancer cell migration toward a spectrum of relevant matrix proteins. Furthermore, the activation state of αvβ3 determines the efficiency of breast cancer cell migration toward certain substrates, such as vitronectin and fibronectin. Most importantly, the receptor activation state determines the ability of the tumor cells to recognize fibrinogen/fibrin as a migration substrate. Breast cancer cells that express activated αvβ3 produce a soluble factor that promotes fibrinogen directed migration of breast cancer cell variants that express αvβ3 in the non-activated functional form.

4. Activated αvβ3 cooperates with metalloproteinase MMP-9 during support of breast cancer cell migration

We next sought to identify the soluble factor, which is produced by breast cancer cells with activated αvβ3 and which promotes breast cancer cell migration. Precedence in the literature suggests that metalloproteinases may be involved [17]. Furthermore, it had been shown that integrin αvβ3 can bind metalloproteinase protein [18,19]. Therefore, we reasoned that integrin αvβ3 could cooperate with a metalloproteinase in the support of breast cancer cell migration. Our results suggest that the activation state of integrin αvβ3 is critical for this cooperation.

We first asked, which metalloproteinases are produced by our breast cancer cell variants. We focused on gelatinases, because the metalloproteinase known to interact with αvβ3 is gelatinase A, also referred to as MMP-2 [19]. By gelatin zymography, we found that all tested variants of our MDA-MB 435 breast cancer cell model produce gelatinases with apparent molecular weights consistent with the latent form of MMP-9 (92 kDa). Significantly, only the breast cancer cell variants expressing activated αvβ3 produced bands at 82 kDa, which are consistent with activated MMP-9 (Fig. 10). All breast cancer cell variants also produced bands at 72 kDa and faint bands at 62 kDa. These are consistent with latent and activated MMP-2, respectively (Fig. 10). The identity of these bands as MMP-9 and MMP-2, respectively, were confirmed by immuno precipitation and western blot analysis. The supernatants of our breast cancer patient derived primary metastatic cell lines BCM-1, BCM-2 and BMS also contained latent and activated MMP-9 (Fig. 11). Thus, the production of activated MMP-9 is associated with the expression of integrin αvβ3 in its activated functional form. This was found in *in vivo* selected and *in vitro* generated variants of our MDA-MB 435 breast cancer cell model and in clinical metastatic breast cancer cells. Based on these results, we propose a model mechanism, in which activated αvβ3 binds the latent 92 kDa form of MMP-9 as a soluble ligand, and thereby facilitates conversion of pro MMP-9 to active MMP-9 at the tumor cell surface (Fig. 12). We further hypothesized that activated MMP-9 is involved in regulating fibrinogen directed migration of metastatic breast cancer cells. This was tested in the following experiments.

To analyze whether the migration inducing factor, produced by breast cancer cells expressing activated αvβ3, is indeed MMP-9, we analyzed if fibrinogen directed migration promoted by this factor is affected by TIMP-1, a natural inhibitor of MMP-9. We found that TIMP-1 significantly reduced fibrinogen directed migration of MDA-MB 435 parental cells (non-activated αvβ3) that was induced by

supernatants from a metastatic variant of the parental cells (bone) or from BCM-2 cells (Fig. 13). TIMP-1 also inhibited fibrinogen directed migration of MDA-MB 435 bone cells and BCM-2 cells (Fig. 13). This indicates that MMP-9 produced by these cells contributes to their migratory activity. To test directly whether MMP-9 indeed promotes fibrinogen directed migration of breast cancer cells that express non-activated αvβ3, we allowed MDA-MB 435 parental cells to migrate in the presence of recombinant MMP-9, adding either the latent 92 kDa enzyme (pro MMP-9) or the activated 82 kDa enzyme. Under the tested conditions, pro MMP-9 had no effect on fibrinogen directed migration of the parental breast cancer cells, but activated MMP-9 significantly enhanced the migratory activity. The increase in migratory activity by activated MMP-9 was comparable to that obtained with supernatant from the bone metastasis derived breast cancer cell variant (Fig. 14). Thus, recombinant active MMP-9, but not latent MMP-9 or either form of MMP-2, triggered fibrinogen directed migration of breast cancer cells that express non-activated αvβ3. We conclude that MMP-9 and tumor cell integrin αvβ3 cooperate in breast cancer cell migration toward a fibrinogen or fibrin matrix. Based on these results, our breast cancer cell variants produce latent MMP-9, regardless of the activation state of their integrin αvβ3. However, only those breast cancer cell variants that express activated αvβ3 are efficient in converting latent to activated MMP-9. The activated enzyme then supports the migratory process.

To analyze how activated MMP-9 may support fibrinogen directed breast cancer cell migration, we asked the question: does MMP-9 modify the fibrinogen matrix? To address this, we used fibrinogen as a substrate in zymography gels. We found that the supernatant of primary metastatic cells from breast cancer patients (shown here is BCM-1) and metastasis derived variants of the MDA-MB 435 breast cancer cell model produce an 82 kDa band, that degrades fibrinogen (Fig. 15). This is consistent with activated MMP-9 (82 kDa). To test directly, whether MMP-9 degrades fibrinogen under these experimental conditions, we analyzed the recombinant enzyme and confirmed that MMP-9 indeed digests a fibrinogen substrate. Activated MMP-9 comigrates with the fibrinogenolytic band from supernatants of metastatic breast cancer cells (Fig. 16). Thus, it is conceivable that the supernatants of breast cancer cells, that express activated αvβ3, contain active MMP-9, which specifically modifies a fibrinogen or fibrin matrix. Collectively this resulted in αvβ3 mediated migration of metastatic breast cancer cells toward this specific substrate.

From the studies completed during the funding period, we conclude that integrin activation, especially activation of integrin αvβ3 and proteolytic activity are functionally related in breast cancer cells. Expression of activated αvβ3 promotes breast cancer cell arrest during blood flow by supporting tumor cell interaction with platelets. Furthermore, the activated receptor mediates breast cancer cell migration toward specific matrices, that are relevant during the escape of tumor cells from the primary tumor stroma and invasive penetration of the vessel wall. Thus integrin αvβ3 activation contributes to adhesive, migratory and potentially invasive tumor cell functions, that are critical during breast cancer metastasis.

5. In our analyses of the modulation of integrin αvβ3 function during metastatic progression, we found two mutations in the integrin β3 subunit genes of highly metastatic variants of our MDA-MB 435 breast cancer cell model. The mutations are located in regions of the molecule that are important for β3 integrin ligand binding functions.

To understand mechanisms that modulate the functional activation state of integrin αvβ3 in metastatic breast cancer cells, we hypothesized that mutations within the αv or β3 subunit genes may play a role. This concept is supported by our finding that expression of mutant $\beta3_{D723R}$ in a β3-minus variant of the MDA-MB 435 parental cells lead to functionally activated integrin αvβ3, as the receptor in the transfected cells was able to bind soluble ligand without external stimulation, supported tumor cell interaction with platelets during blood flow and thereby mediated tumor cell arrest. Most importantly, activated αvβ3 induced a highly metastatic phenotype in the transfected cells. To probe for naturally occurring receptor mutations in our MDA-MB 435 cell model, we started with sequencing the full length β3 subunit genes of MDA-MB 435 parental cells (non-activated αvβ3, low metastatic activity) and their *in vivo* selected variants from a lung metastase. We found several point mutations, several of which lead to amino acid changes in the β3 subunit protein. Importantly, all of these mutations are located in positions of the protein that are know to affect the ligand binding activity of β3 integrins:

Cell variant	nucleotide change	amino acid change	region/comment
MDA-MB 435 lung	T 177 C	S 77 P	N-terminus/abolishes *HgiAI* restriction site
MDA-MB 435 lung	G 2037 C	D 647 H	close to transmembrane region, extracellular side

To analyze whether these β3 mutations affect the functional activation state of breast cancer cell integrin αvβ3, we have now established the following mutations in our β3 gene vector system by site directed mutagenesis: $\beta3_{T177C}$, $\beta3_{G2037C}$, and the double mutant $\beta3_{T177C+G2037C}$. In the coming funding period, we will use these mutants for stable transfection of the β3-minus MDA-MB 435 cell variant and functional analysis of the resulting αvβ3 receptor expressed in these cells.

Together, our results from the second year of funding respond to and extend beyond the originally proposed tasks in the statement of work:

Task 1. Analyze the metastatic potential of adhesive variants of the MDA-MB 435 human breast cancer cell line that express the activated or the resting form of integrin αvβ3.
(This task was completed during the first year of funding)

Task 2. Analyze the modulation of integrin αvβ3 function in adhesive variants of MDA-MB 435 breast cancer cells.
In the first year of funding, we found that a mutant β3, namely $\beta3_{D723R,}$ can result in the constitutively activated αvβ3 receptor. Forced expression of this β3 mutant in our β3-minus MDA-MB 435 breast cancer cell variant resulted in a changed adhesive tumor cell phenotype and, most importantly, in a highly metastatic cell variant.
We now report that the naturally selected metastatic variant of the parental MDA-MB 435 cell line, established from a lung metastase in SCID mice, contained β3 subunit gene mutations, which are located in functionally relevant portions of the integrin β chain. Potential association of breast cancer cell integrin αvβ3 with accessory proteins that may modulate the receptor function, well be analyzed in the

upcoming funding period.

Task 3. Test the significance of integrin αvβ3 activation in human breast cancer
In the fist funding year, we had established a panel of primary metastatic cells from pleural effusions and peripheral blood samples of patients with stage IV breast cancer. We had also found that these cells expressed integrin αvβ3, and that the receptor was present in a constitutively activated functional form, as judged by the platelet-interactive and arrest competent phenotype of the cells.
We now report that these primary metastatic cells, as well as the *in vivo* selected and the mutant $\beta3_{D723R}$ expressing *in vitro* generated variant of our MDA-MB 435 breast cancer cell model exhibit adhesive and migratory activities that support the metastatic phenotype of these cells. The finding that activated integrin αvβ3 is functionally linked to metalloproteinase activation in these cells, and that αvβ3 and activated MMP-9 cooperate in breast cancer cell migration is a new finding that was not originally considered. This new finding opens a novel line of investigation, which will help to understand how activated integrin αvβ3 controls metastasis in human breast cancer.

KEY RESEARCH ACCOMPLISHMENTS:

- We established primary metastatic tumor cells from malignant effusions and peripheral blood samples of patients with stage IV breast cancer, and found that these cells express integrin αvβ3 in its activated functional state

- We tested one of our primary metastatic cell lines, established from a blood sample of a patient with metastatic breast cancer, for metastatic activity in a SCID mouse model and found that the cell line BCM-2 serves as a new cell model for studies of human breast cancer in immune deficient mice

- We found that activated integrin αvβ3 promotes the metastatic phenotype in human breast cancer cells by supporting and enhancing breast cancer cell migration toward extracellular matrix proteins

- We found that activated integrin αvβ3 cooperates with metalloproteinase MMP-9 during support of breast cancer cell migration

- In our analyses of the modulation of integrin αvβ3 function during metastatic progression, we found two mutations in the integrin β3 subunit genes of highly metastatic variants of our MDA-MB 435 breast cancer cell model. The mutations are located in regions of the molecule that are important for β3 integrin ligand binding functions

REPORTABLE OUTCOMES:

Publications:

- Felding-Habermann B, O'Toole TE, Smith JW, Fransvea E, Ruggeri ZM, Ginsberg MH, Hughes PE, Pampori N, Shattil SJ, Saven A, and Bueller BM (2001) Integrin activation controls metastasis in human breast cancer. Proc. Natl. Acad. Sci. USA 98: 1853-1858

- Tumor cell-platelet interaction in metastatic disease (2001) Felding-Habermann B, Haemostasis 31, S1: 55-58

Abstracts:

- Unique ability of integrin αvβ3 to support tumor cell arrest under dynamic flow conditions (2001) Pilch J, and Felding-Habermann B. 2001 Meeting of the American Society of Hematology, Orlando Florida

- Activated integrin αvβ3 and metalloproteinase MMP-9 cooperate in migration of metastatic breast cancer cells (Submitted) Rolli M, Fransvea E, Pilch J, Saven A, and Felding-Habermann B. 2002 Annual Meeting of the American Association for Cancer Research, San Francisco 2002

- Expression of activated integrin αvβ3 in clinical breast cancer metastasis (Submitted) Rolli M, Saven A, Russack V, Pilch J and Felding-Habermann B. 2002 Annual Meeting of the American Society of Clinical Oncology, Orlando 2002

Presentations:

- The platelet interactive and metastatic phenotype in tumor metastasis. Invited lecture at the Burnham Institute, La Jolla, CA, March 5th, 2001

- Role of integrin activation in breast cancer cell invasion and migration. Invited lecture at the Novartis Summit Meeting, Summit, New Jersey, August 14th, 2001

- Integrin activation in tumor metastasis. Invited lecture. The Scripps Research Institute Vascular Biology Affinity Group, La Jolla, CA, October 19th, 2001

- Tumor cell- platelet interaction in metastatic disease. Invited lecture at the First International Conference on Thrombosis and Haemostasis Issues in Cancer, Bergamo, Italy, November 4th, 2001

- Control of breast cancer cell migration and invasion. Oral presentation at the Annual Retreat of the Department of Molecular and Experimental Medicine, The Scripps Research Institute, San Diego, CA, November 7th, 2001

New cell model for studies of human breast cancer metastasis in immune deficient mice

β3 mutant vector constructs: pc DNA 1-neo containing full length human $\beta3_{T177C}$ or $\beta3_{G2037C}$ or the double mutant $\beta3_{T177C + G2037C}$

Funding applied for based on work supported by this award

Integrin Activation in Breast Cancer Metastasis. PI: Brunhilde Felding-Habermann, Funding agency: National Institutes of Health, Award type: R01 Research Grant. Grant number R01 CA95458-01

CONCLUSIONS:

From the results generated during the past funding period, we conclude that human breast cancer cells can express the adhesion receptor integrin αvβ3 in an activated or a non-activated functional form. The activation state of the receptor had a profound impact on the metastatic activity of the breast cancer cells, in that the activated, but not the non-activated receptor supports metastatic dissemination. Mechanisms through which activated integrin αvβ3 are likely to promote metastasis are: binding of soluble ligand, support of tumor cell arrest during blood flow based on tumor cell interaction with platelets, enhancement and specific promotion of breast cancer cell migration toward matrix proteins, that are relevant during metastatic dissemination. The mechanism through which activated integrin αvβ3 promotes breast cancer migrtion depends on a functional cooperation between activated integrin αvβ3 and metalloproteinase MMP-9. A model mechanism for the functional cooperation between the adhesion receptor an the metalloproteinase is proposed. Together, our new findings support our originally proposed concept, that activation of breast cancer cell integrin αvβ3 supports the metastatic phenotype in the tumor cells. Thus, our studies identify activated αvβ3 as a new functional marker of metastatic breast cancer cells. Alterations within tumors that lead to the aberrant control of αvβ3 activation are expected to adversely affect the course of human breast cancer. Our studies indicate that activated αvβ3 should be considered as a new functional target for the inhibition of breast cancer metastasis.

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APPENDICES:

Figure legends and Figures

Publications

Figure legends

Figure 1. Isolation of circulating breast cancer cells. Circulating metastatic cells were isolated from peripheral blood samples of patients with stage IV breast cancer. Blood samples were incubated with supra immuno magnetic beads that were decorated with monoclonal antibody Ber EP-4. Mab Ber EP-4 recognizes a human epithelial cell surface antigen. The washed beads were treated with release buffer to release potentially bound tumor cells and taken into sterile culture. Surviving tumor cells were expanded, frozen and used in functional studies, as soon as sufficient cell numbers were accumulated.

Figure 2. Primary metastatic cells from breast cancer patients express activated αvβ3. The primary metastatic human breast cancer cell lines BCM-1, BCM-2 and BMS were analyzed for integrin αvβ3 expression by flow cytometry (left panel). The functional activation state of αvβ3 expressed by these cells was tested in blood perfusion studies (right panel). The tumor cells were prestainded with hydroethidine, mixed into whole human blood and perfused over a thrombogenic collagen I matrix at a venous wall shear rate (50 sec^{-1}). During ongoing perfusion, adhesive events were recorded by real-time video fluorescence microscopy. Without interrupting the flow, images were captured at 50 predefined *x,y* positions after a defined time period. Two sets of images were captured at each positions: one with filter settings to detect platelets and one with filter settings to detect tumor cells. Digital image processing revealed that the tumor cells did not arrest directly on the collagen matrix, but were associated with adherent platelets. Perfusion experiments in the presence of function blocking anti-αvβ3 mab VNR1.27.1 (80 μg/ml) showed that tumor cell arrest was strongly reduced when tumor cell αvβ3 was blocked. Also, blocking of platelet integrin αIIbβ3 resulted in an almost complete loss of tumor cell arrest. Thus, primary metastatic breast cancer cells utilize αvβ3 for platelet dependent tumor cell arrest during blood flow. This indicates that the adhesion receptor is in a functionally activated state.

Figure 3. BCM-2 cells, isolated from breast cancer patient blood, serve as a new model for human breast cancer metastasis in SCID mice. BCM-2 cells ($1x10^6$) cells were injected into the lateral tail vein of 8 week old female CB17 SCID mice. Six weeks later, the animals were sacrificed, and metastatic colonization was evaluated at the surface of the lungs. The left lung is from an animal injected with BCM-2 cells. Metastatic foci are clearly visible at the lungs surface. The right lung is from a healthy control mouse.

Figure 4. Activated αvβ3 mediates and enhances breast cancer cell migration. Breast cancer cell variants, that express integrin αvβ3 either in an activated or a non-activated functional form, were allowed to migrate toward extracellular matrix proteins coated to the underside of porous filters in modified Boyden chambers (Transwell chambers with 8 μm pores). Serum free EMEM culture medium was used as migration buffer. The chambers were incubated for 16 hrs at 37C and 5% CO_2. After the

migration period, the filter inserts were washed gently, excised, fixed and stained with DiffQuick. Migrated cells were counted at the underside of the filters. The data represent the number of migrated cells within 5 microscopic fields. Each condition was tested in duplicate or triplicate, and 5 optical fields were counted per filter. The cell variants used were: Parent (MDA-MB 435 parental cells, express non-activated αvβ3), Bone (variant of Parent derived from a bone metastase after injecting Parent into the mammary fat pad of a SCID mouse), β3minus (β3 lacking variant of Parent), $\beta3_{WT}$ (β3 minus after transfection with wild type β3, express non-activated αvβ3), $\beta3_{D723R}$ (β3 minus after transfection with the constitutively activating β3 mutant $\beta3_{D723R}$), BCM1, BCM2 and BMS (primary metastatic cells isolated from peripheral blood of patients with stage IV breast cancer, express activated αvβ3), PE02JA (primary metastatic cells from a malignant effusion of a breast cancer patient, express activated αvβ3). Matrix proteins were vitronectin (VN), fibrinogen (Fg) and von Willebrand Factor (vWF).

Figure 5. Activated αvβ3 mediates breast cancer cell migration toward fibrinogen. Breast cancer cells were allowed to migrate toward a fibrinogen substrate in the presence or absence of function blocking anti β3 mab 7E3 (80 μg/ml). The general experimental conditions were as in Fig. 4.

Figure 6. Activated αvβ3 mediates fibrinogen directed breast cancer cell migration without exogenous stimuli. Breast cancer cells were allowed to migrate toward a fibrinogen substrate in the absence of exogenously added manganese (Mn^{2+}) (left panes) or in the presence of increasing concentrations of manganese. The general experimental conditions were as in Fig. 4.

Figure 7. Supernatants of breast cancer cells expressing activated αvβ3 enhance tumor cell migration. Breast cancer cells were allowed to migrate toward a fibrinogen matrix either in medium without supplements (as in Fig. 4) or in conditioned serum free supernatant harvested from a 16 hr culture of the same cell type (own sup), or of other breast cancer cell variants. Otherwise, the general experimental conditions were as in Fig. 4.

Figure 8. Supernatant enhanced breast cancer cell migration is mediated by αvβ3. MDA-MB 435 parental cells were allowed to migrate toward a fibrinogen matrix either in medium as in Fig. 4, conditioned supernatant of the same cell type or other breast cancer cell variants either in the presence or absence of function blocking anti β3 mab 7E3 (80 μg/ml). The general experimental conditions were as in Fig. 4.

Figure 9. Supernatants of breast cancer cells expressing activated αvβ3 enhance tumor cell migration in a substrate specific manner. Breast cancer cell variants were allowed toward vitronectin (VN) or fibronectin (FN) matrices as in Fig. 7. The general experimental conditions were as in Fig. 4.

Figure 10. Activation of integrin αvβ3 and metalloproteinase MMP-9 are associated in MDA-MB 435 breast cancer cells. Concentrated, serum free supernatants of MDA-MB 435 breast cancer cell variants were analyzed by gelatin zymography. Serum free supernatants of the breast cancer cell variants were harvested from 48 hr cultures, concentrated by $(NH_4)_2SO_4$ precipitation, dialyzed and run on 10% polyacrylamide gels containing 0.05% gelatin under non-reducing conditions. The gels were washed with Triton-X100 containing buffer and incubated in substrate buffer over night at 37C. Gelatinolytic bands were visualized after staining the gels with Coomassie blue and brief destaining. The tumor cell variants were: HT1080 human fibrosarcoma cells (positive control), and MDA-MB 435 cell variants as in Fig. 4. Lung represents an MDA-MB 435 cell variant isolated from a lung metastase, after injecting the parental cells into the mammary fat pad of a SCID mouse. Note: $\beta3_{D723R}$ expresses activated αvβ3, Lung expresses activated αvβ3, β3 minus lacks αvβ3 expression, and $\beta3_{WT}$ and Parent express non-activated αvβ3.

Figure 11. Activation of integrin αvβ3 and metalloproteinase MMP-9 are associated in metastatic cells from breast cancer patients. Gelatin zymography of concentrated supernatants harvested from BCM-1, BCM-2 or BMS primary metastatic cells from breast cancer patients. The general experimental conditions were as in Fig. 10.

Figure 12. Model for MMP-9 activation on breast cancer cells.

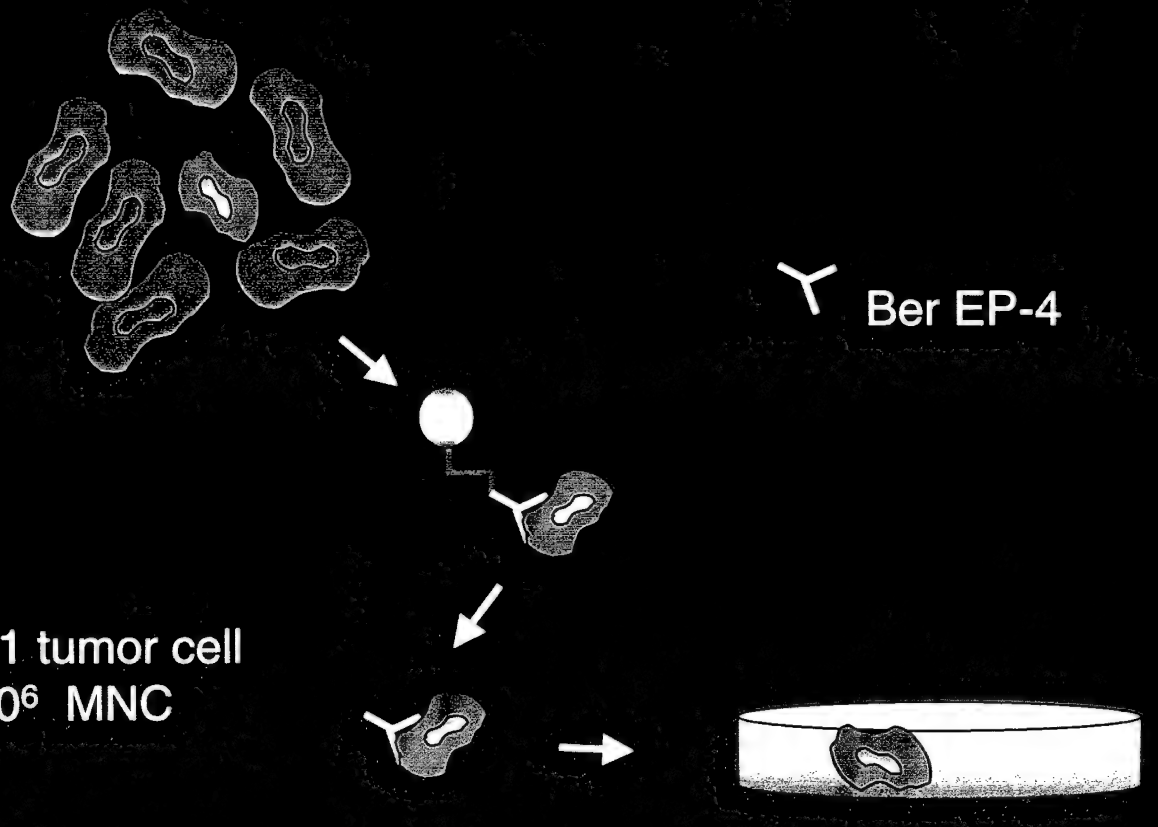
Figure 13. TIMP-1 inhibits supernatant enhanced breast cancer cell migration. Breast cancer cell variants were allowed to migrate toward a fibrinogen matrix in the presence or absence of 20 ng/ml TIMP-1. The general experimental conditions were as in Fig. 4, spent supernatants were used as migration buffer as in Fig. 7.

Figure 14. Active MMP-9 enhances breast cancer cell migration. MDA-MB 435 parental cells (non-activated αvβ3) were allowed to migrate toward a fibrinogen matrix in the presence or absence of exogenously added recombinant pro MMP-2, proMMP-9, active MMP-2 or active MMP-9, or in spent supernatant from the MDA-MB 435 cell variant isolated from a bone metastase. General experimental conditions were as in Fig. 4.

Figure 15. Fibrinogenolytic activity in supernatants of metastatic breast cancer cells. Concentrated serum free supernatants from breast cancer cell variants were analyzed by fibrinogen zymography. The general experimental conditions were as in Fig. 10, except that fibrinogen was incorporated into the gels instead of gelatin.

Figure 16. Fibrinogenolytic activity in supernatants of metastatic breast cancer cells is related to MMP-9. Fibrinogen zymography with concentrated spent supernatants of MDA-MB 435 parental cells or their bone metastasis derived variant, or with recombinant latent (pro) or activated MMP-2 or MMP-9. The general experimental conditions were as in Fig. 10 and 15.

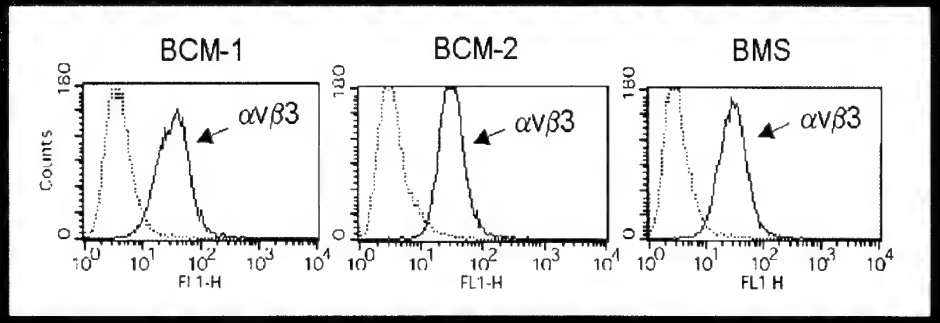
Isolation of circulating breast cancer cells



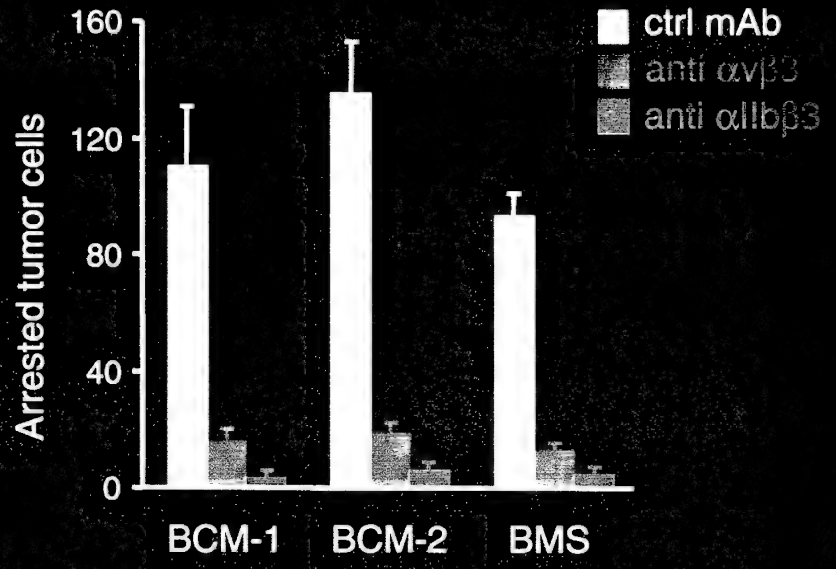
Established primary metastatic cells: BCM1, BCM2, BMS

B. Felding-Habermann Figure 1

Primary metastatic cells from breast cancer patients express activated αvβ3



Platelet dependent tumor cell arrest during blood flow

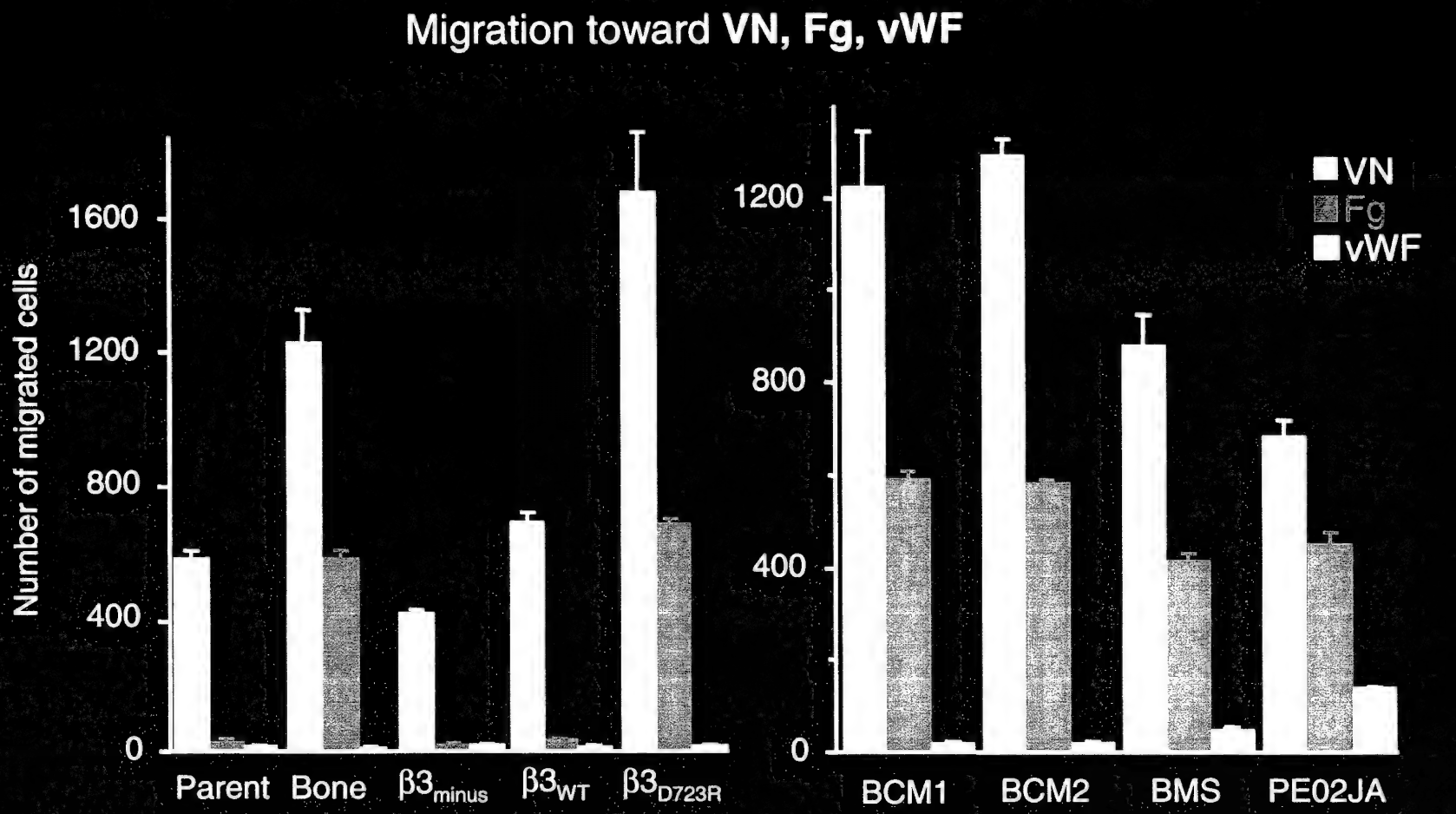


B. Felding-Habermann Figure 2

BCM2 cells, isolated from breast cancer patient blood, serve as model for human breast cancer in scid mice

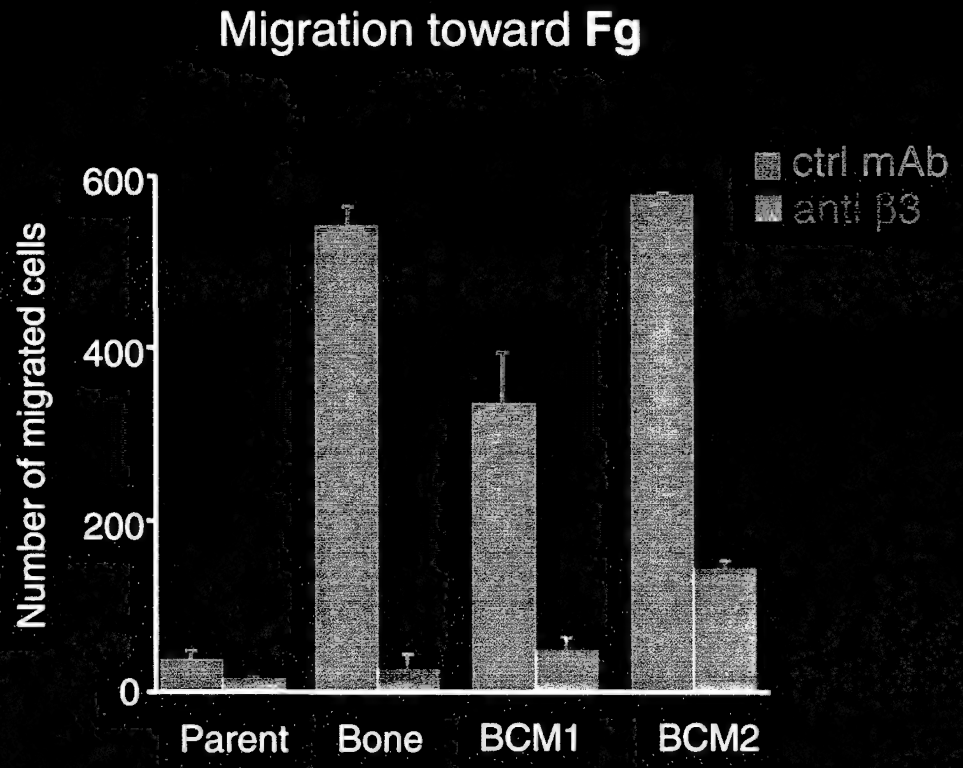
B. Felding-Habermann Figure 3

Activated αvβ3 mediates and enhances breast cancer cell migration



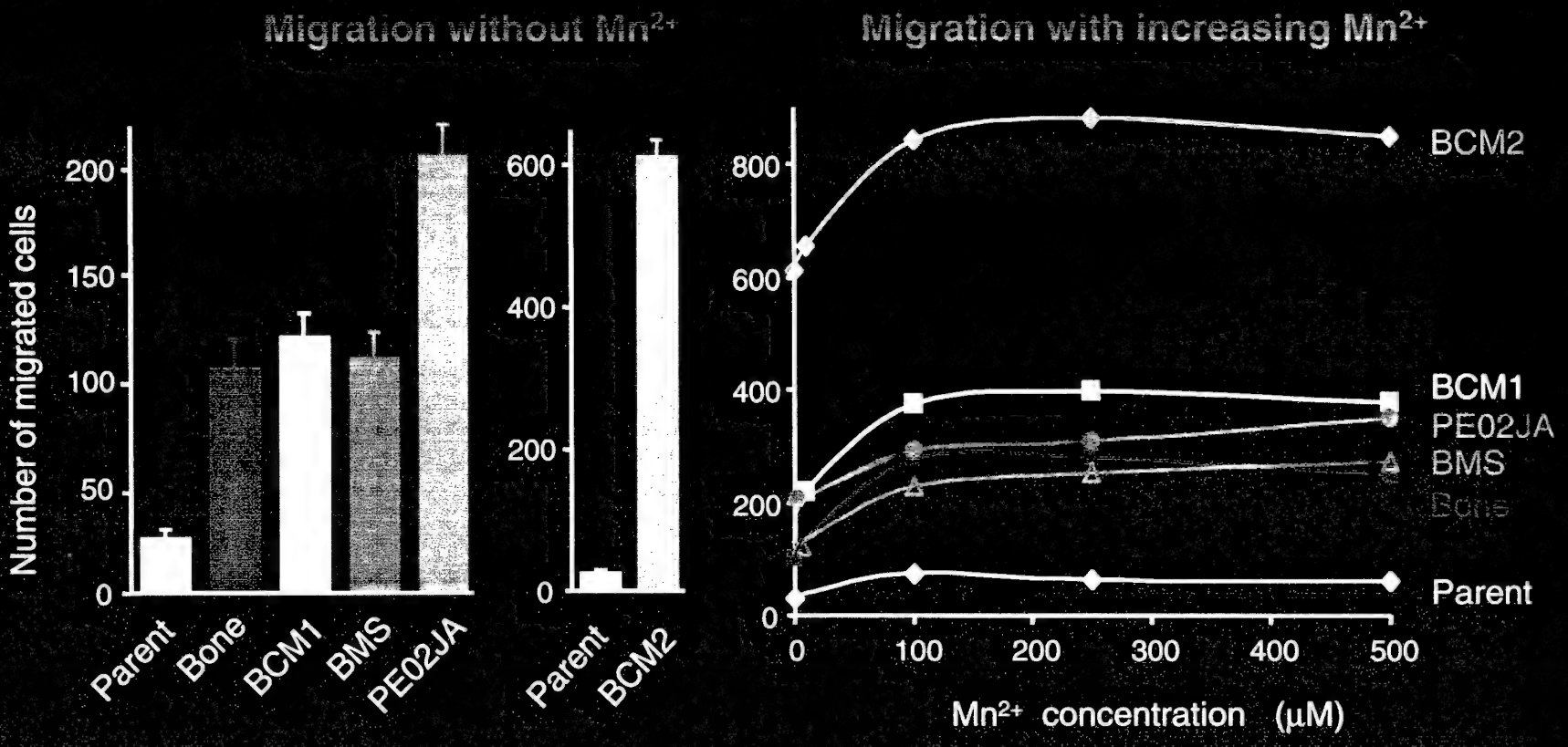
B. Felding-Habermann Figure 4

Activated αvβ3 mediates breast cancer cell migration



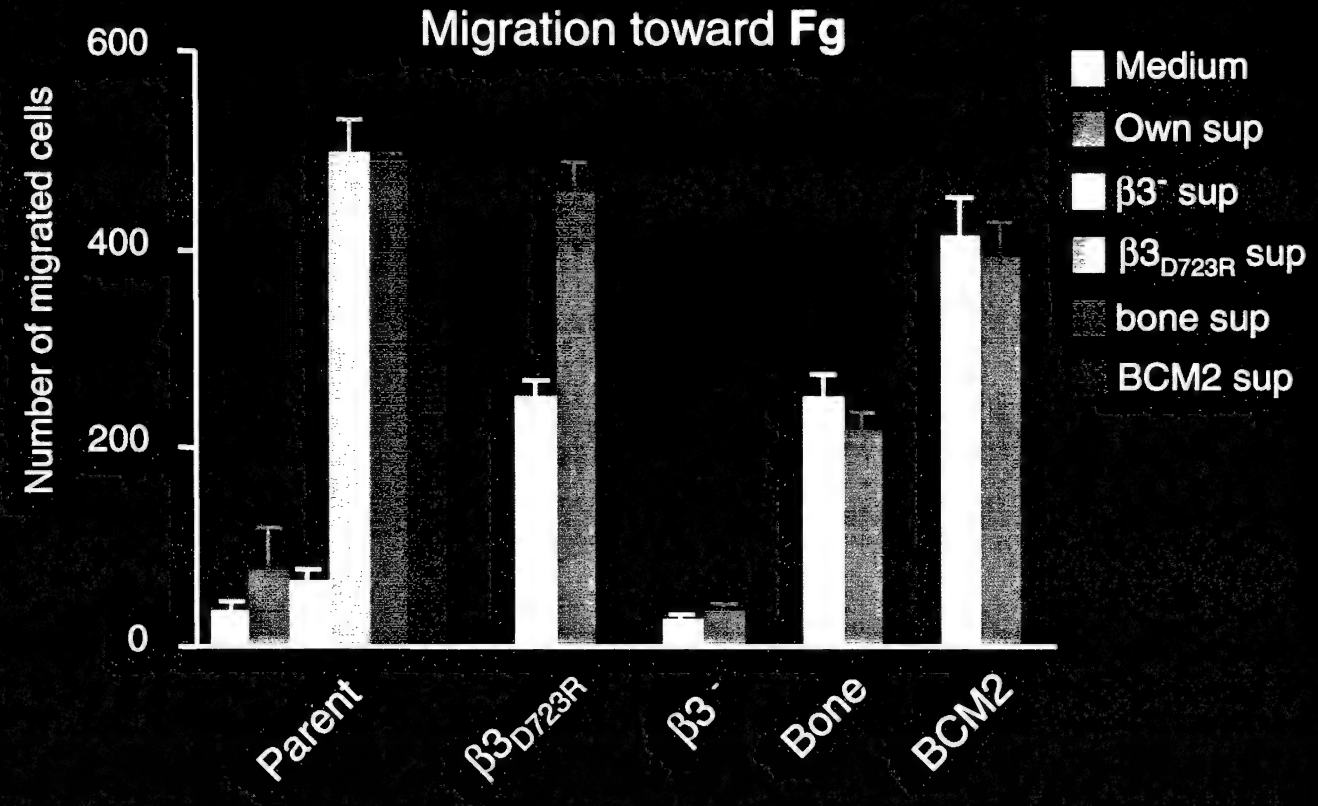
B. Felding-Habermann Figure 5

Activated αvβ3 mediates Fg directed breast cancer cell migration without exogenous stimuli



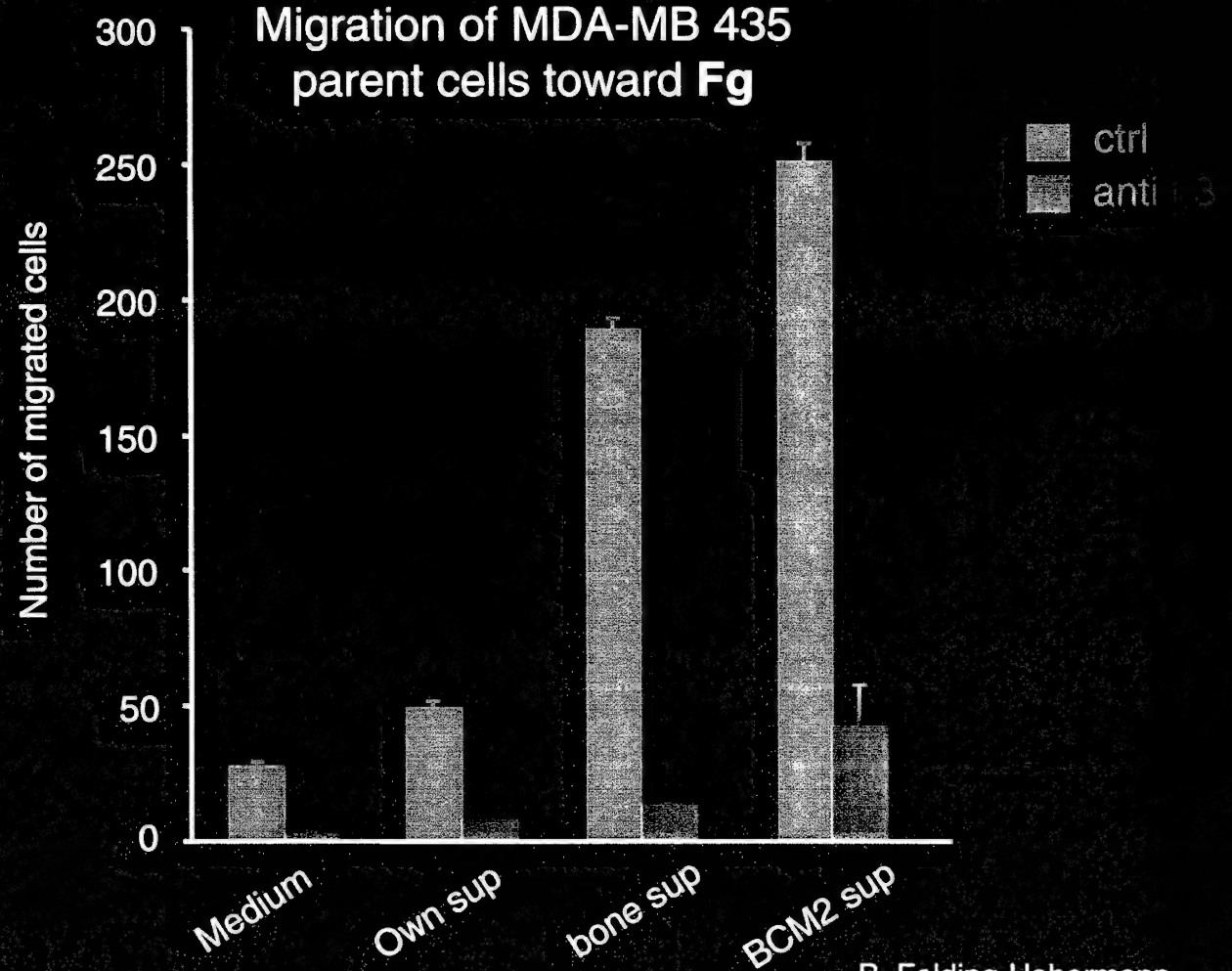
B. Felding-Habermann Figure 6

Supernatants of breast cancer cells expressing activated αvβ3 enhance tumor cell migration



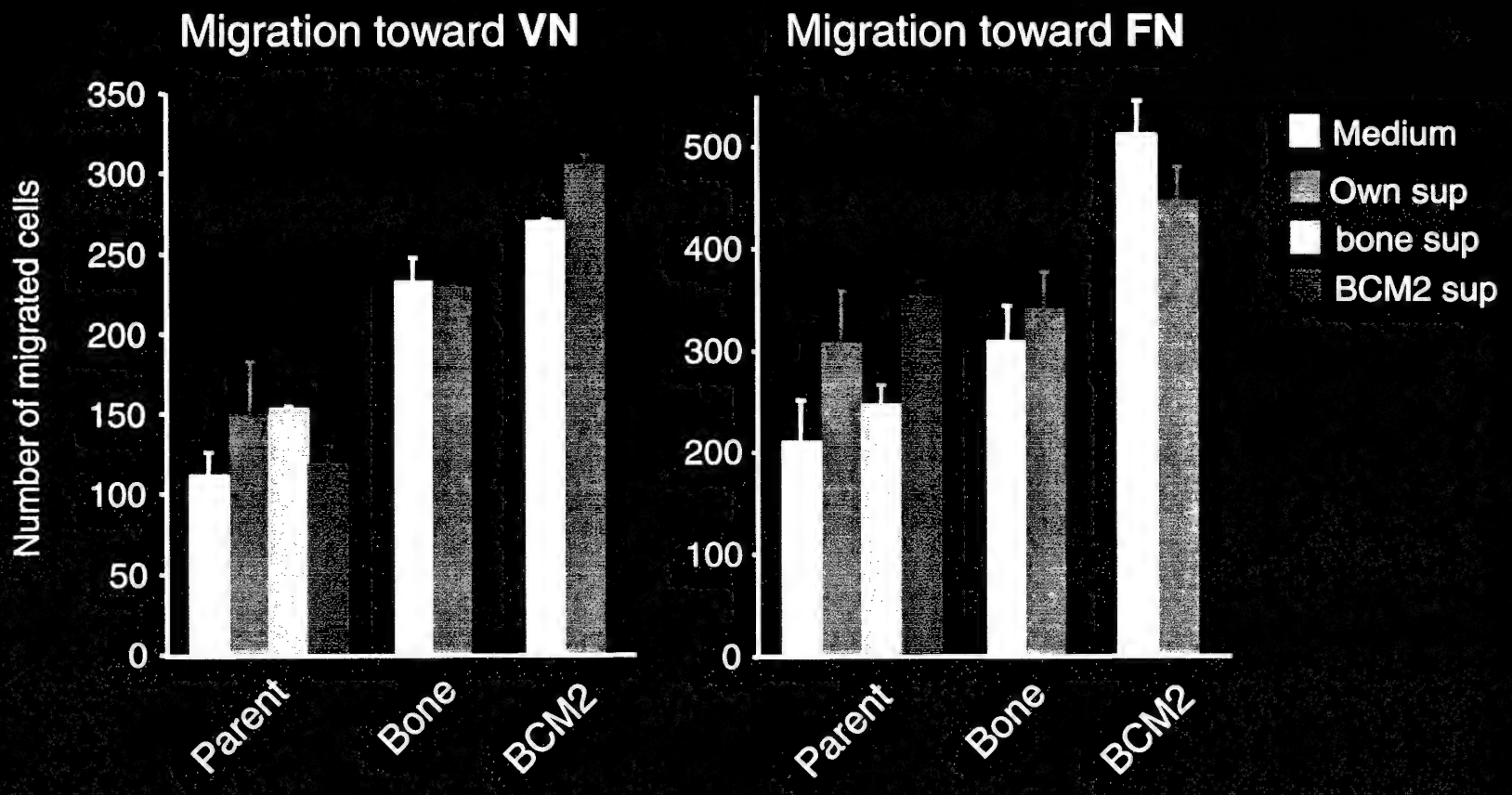
B. Felding-Habermann Figure 7

Supernatant enhanced breast cancer cell migration is mediated by αvβ3



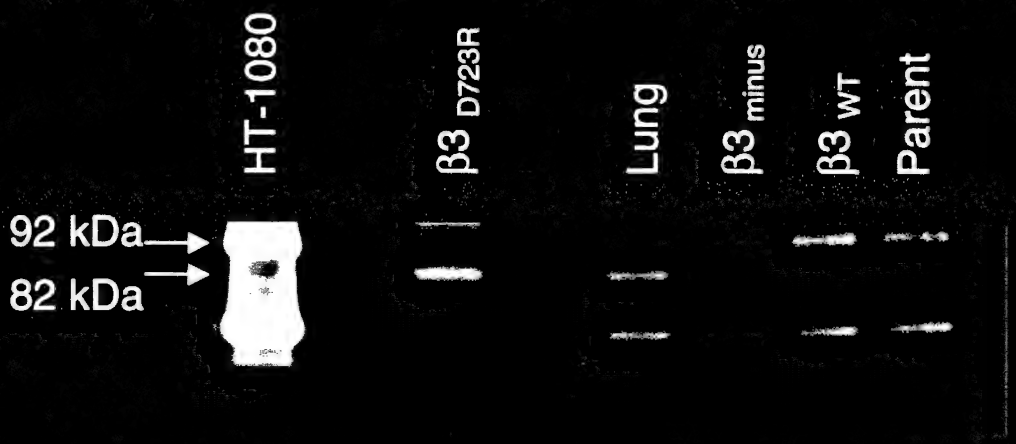
B. Felding-Habermann Figure 8

Supernatants of breast cancer cells expressing activated αvβ3 do not enhance tumor cell migration toward all substrates



B. Felding-Habermann Figure 9

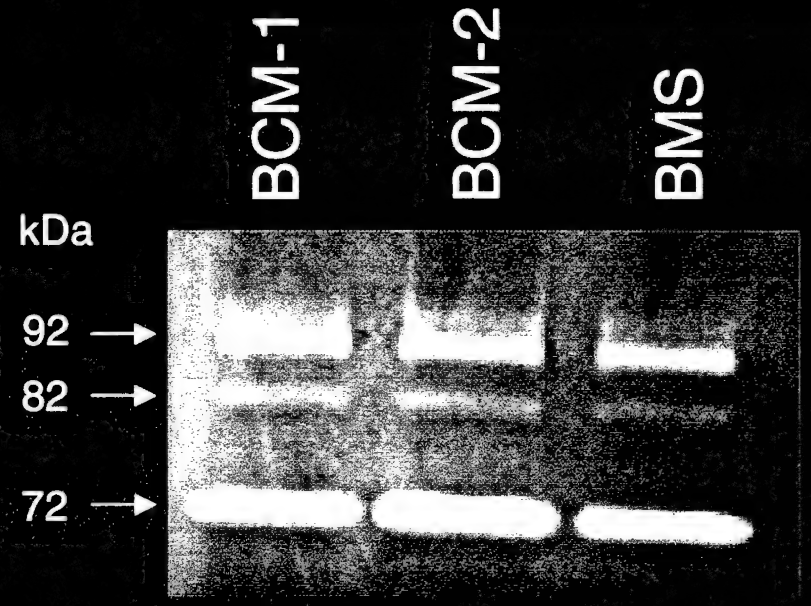
Activation of MMP-9 and αvβ3 correlate in MDA-MB 435 breast cancer cells



Gelatin zymogram of concentrated supernatants

B. Felding-Habermann Figure 10

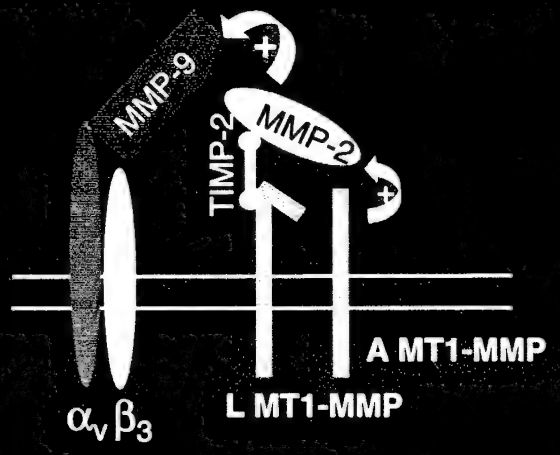
Activation of MMP-9 and αvβ3 correlate in Metastatic cells from Breast Cancer Patients

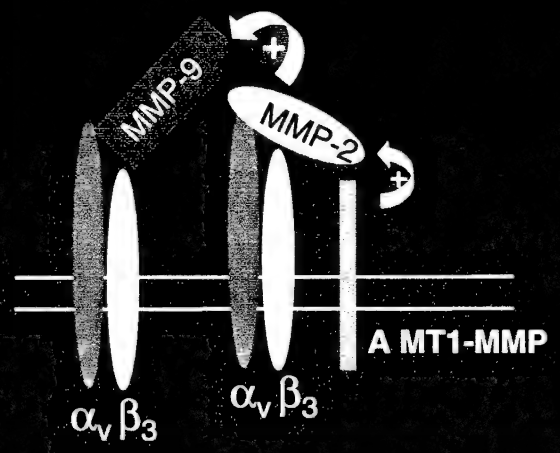


Gelatin zymogram of concentrated supernatants

B. Felding-Habermann Figure 11

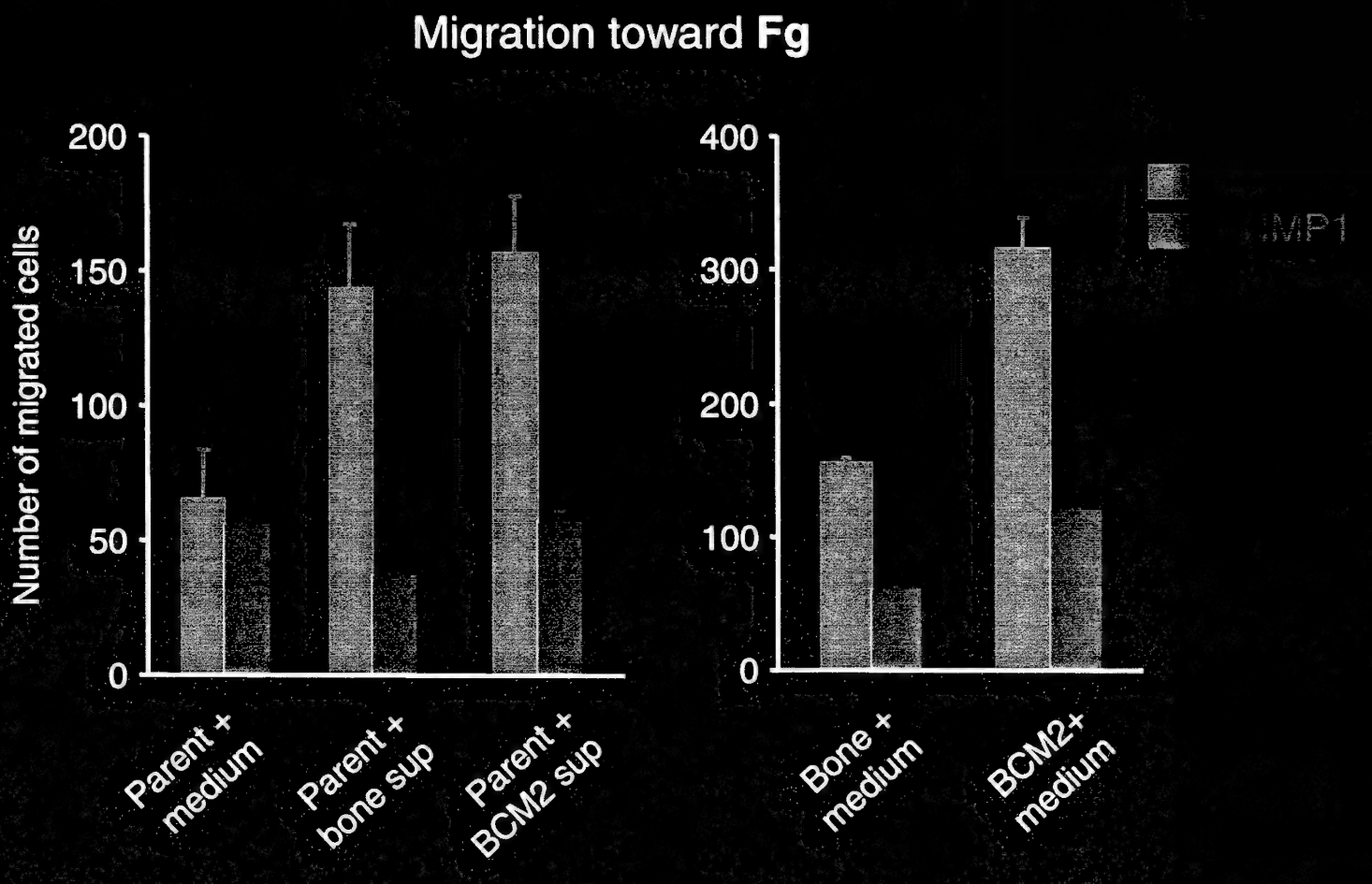
Model for MMP-9 Activation on Breast Cancer Cells





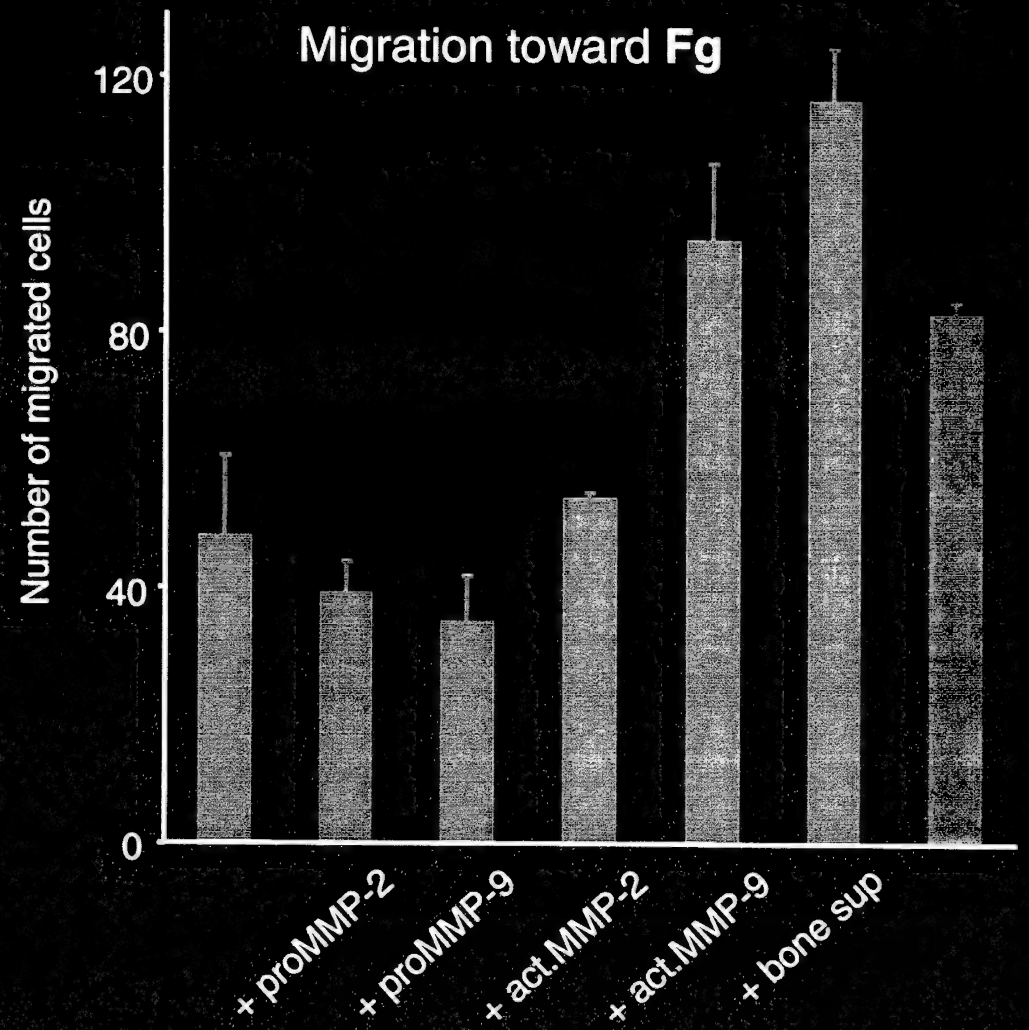
B. Felding-Habermann Figure 12

TIMP-1 inhibits supernatant enhanced breast cancer cell migration



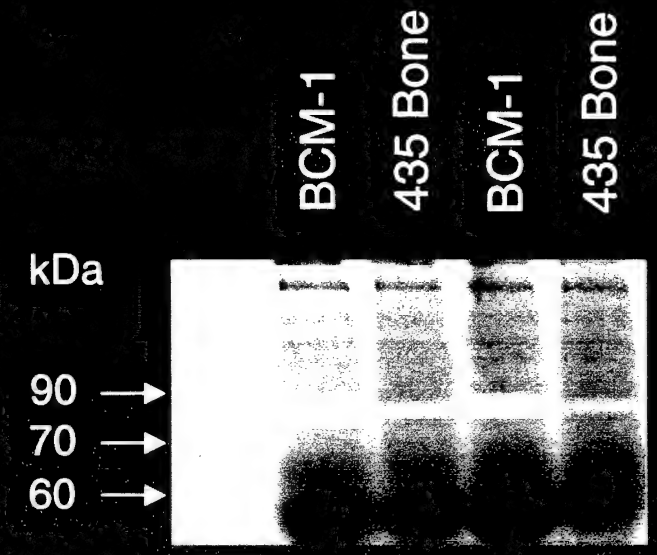
B. Felding-Habermann Figure 13

Active MMP-9 enhances breast cancer cell migration



B. Felding-Habermann Figure 14

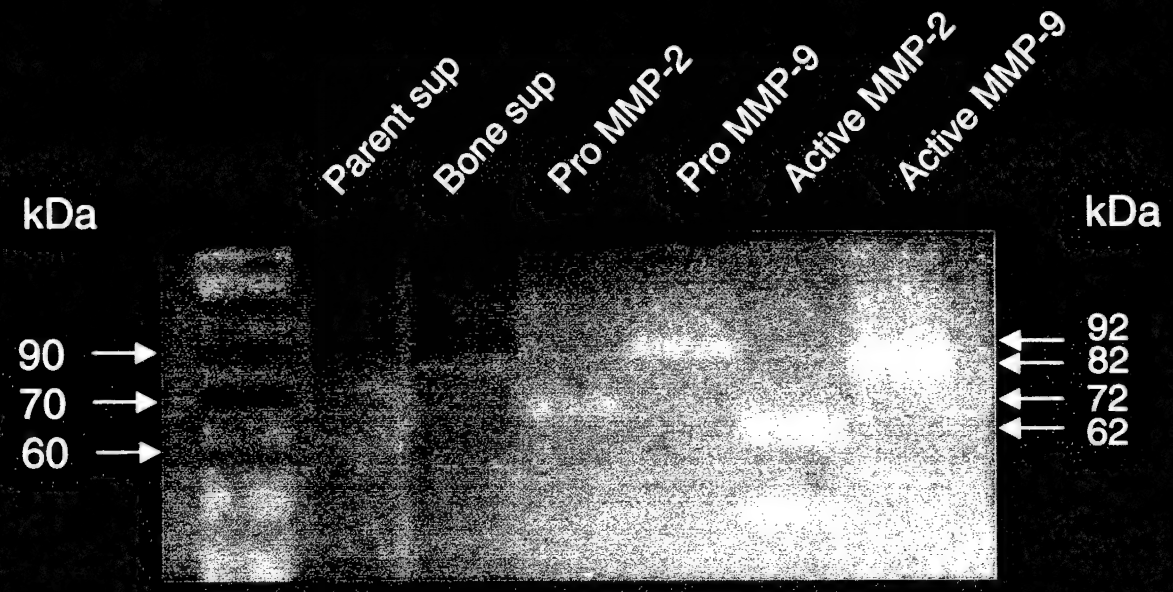
Fibrinogenolytic activity in supernatants of metastatic breast cancer cells



Fibrinogen zymogram of concentrated supernatants

B. Felding-Habermann Figure 15

Fibrinogenolytic activity in supernatants of metastatic breast cancer cells is related to MMP-9



Fibrinogen zymogram of concentrated supernatants/recombinant MMPs

B. Felding-Habermann Figure 16

Integrin activation controls metastasis in human breast cancer

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Metastasis is the primary cause of death in human breast cancer. Metastasis to bone, lungs, liver, and brain involves dissemination of breast cancer cells via the bloodstream and requires adhesion within the vasculature. Blood cell adhesion within the vasculature depends on integrins, a family of transmembrane adhesion receptors, and is regulated by integrin activation. Here we show that integrin $\alpha v\beta 3$ supports breast cancer cell attachment under blood flow conditions in an activation-dependent manner. Integrin $\alpha v\beta 3$ was found in two distinct functional states in human breast cancer cells. The activated, but not the nonactivated, state supported tumor cell arrest during blood flow through interaction with platelets. Importantly, activated $\alpha v\beta 3$ was expressed by freshly isolated metastatic human breast cancer cells and variants of the MDA-MB 435 human breast cancer cell line, derived from mammary fat pad tumors or distant metastases in severe combined immunodeficient mice. Expression of constitutively activated mutant $\alpha v\beta 3_{D723R}$, but not $\alpha v\beta 3_{WT}$, in MDA-MB 435 cells strongly promoted metastasis in the mouse model. Thus breast cancer cells can exhibit a platelet-interactive and metastatic phenotype that is controlled by the activation of integrin $\alpha v\beta 3$. Consequently, alterations within tumors that lead to the aberrant control of integrin activation are expected to adversely affect the course of human breast cancer.

Complications from metastatic disease are the primary cause of death in breast cancer. Metastasis to bone, lungs, liver, and brain involves dissemination of tumor cells via the bloodstream (1). This process depends on tumor cell intravasation, adhesion to the vessel wall, extravasation, infiltration, and proliferation into target tissue. Many of these steps involve integrins, a family of transmembrane adhesion receptors composed of noncovalently linked α and β subunits (2). Integrins are known to exist in distinct activation states, which exhibit different affinities for ligand. In general, integrin activation controls cell adhesion (3). Such control is particularly important in the vasculature, where dynamic flow physically opposes cell attachment.

Integrin $\alpha v\beta 3$ has been implicated in the pathophysiology of malignant tumors. It plays a role on endothelial cells, where it is required for tumor angiogenesis (4). In several malignancies, however, the tumor cells express $\alpha v\beta 3$, and this expression correlates with tumor progression in melanoma, glioma, and ovarian and breast cancer (5–8). In breast cancer, $\alpha v\beta 3$ characterizes the metastatic phenotype, as this integrin is upregulated in invasive tumors and distant metastases (9). However, a mechanistic role of $\alpha v\beta 3$ in breast cancer spread has yet to be established. We suggested that an interaction of circulating tumor cells with platelets represents a potential mechanism for tumor cell arrest within the vasculature (10). During blood flow, shear forces oppose cell attachment. Therefore, cells must be equipped with specific adhesive mechanisms to support cell arrest (11). Intravascular attachment of leukocytes and platelets during inflammation and thrombus formation is tightly regulated and depends on integrin activation (12–14). It is unknown whether integrin activation controls tumor cell arrest in a similar manner. Here we provide evidence that activation of integrin $\alpha v\beta 3$ promotes breast cancer cell arrest during blood flow and controls the metastatic activity. Consequently, alterations within tumors that support integrin activation are expected to adversely affect the course of human breast cancer.

Materials and Methods

Matrix Proteins. Bovine fibrillar collagen I (Sigma) was used as a thrombogenic matrix in blood perfusion (10). Vitronectin and fibrinogen were purified from human plasma by affinity (15) or gel filtration (16) chromatography. Human plasma fibronectin was purchased from Collaborative Biomedical Products, Bedford, MA.

Antibodies. All antibodies were murine monoclonal IgGs except WOW-1, a recombinant Fab fragment (17). They were purified on protein A. mAb VNR1 27.1 (function blocking anti-$\alpha v\beta 3$) (18) served to test $\alpha v\beta 3$-mediated cell adhesion. mAb 15 (anti-$\beta 3$) (18) was conjugated to saporin to select $\beta 3$-negative breast cancer cells. mAbs LM609 (anti-$\alpha v\beta 3$) (19), Tγ (anti-thyroglobulin) (control IgG), AV-8 (anti-αv), AV-10 (anti-$\beta 3$) (10), 15F11 (anti-$\alpha v\beta 5$) (20), and 12F1 (anti-$\alpha 2$) (21) were used to analyze integrin expression.

Cells and Cell Lines. MDA-MB 435 human breast carcinoma cells were from J. E. Price (M. D. Anderson Cancer Center, Houston) (22). We derived variants from this cell line by injecting 5×10^5 MDA-MB 435 parental cells into the mammary fat pad of adult female C.B 17/lcrTac *scid* mice (Taconic Farms). After 8 weeks, tumors were removed, mice were allowed to recover, and tumors were minced and cultured. Three weeks later, mice were killed, and metastases were recovered from bone, lungs, lymph nodes, and the pleural cavity and cultured. PE02JA cells are primary metastatic human breast carcinoma cells isolated from a pleural effusion of a patient with stage IV breast cancer. All cells were grown in Eagle's minimal essential medium plus 10% FBS, pyruvate, L-glutamine, vitamins, and nonessential amino acids (BioWhittaker).

Isolation of $\beta 3$ Integrin-Negative MDA-MB 435 Breast Cancer Cells. A $\beta 3$-lacking MDA-MB 435 variant was isolated by exposing the parental cell line to an anti-$\beta 3$-saporin conjugate (Ab15-Sap) (18, 23). A concentration of Ab 15-Sap of 1.6 nM killed most cells within 3 days. Surviving cells were grown without toxin for 4 days

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and then analyzed for integrin expression by flow cytometry. Lack of β3-integrin was routinely confirmed during this study.

Transfection. The β3-negative MDA-MB 435 cell variant was transfected with human $\beta3_{WT}$ or mutant $\beta3_{D723R}$ cDNA (24) with the use of Lipofectamine (GIBCO/BRL). Stable transfectants were selected in G418 (1.5 mg/ml for 4 weeks). Integrin expression was monitored by flow cytometry. Cell populations expressing $\alpha v\beta3_{WT}$ or mutant $\alpha v\ \beta3_{D723R}$ at levels comparable to that of αvβ3 in parental MDA-MB 435 cells were collected by sterile FACsorting.

Flow Cytometry. MDA-MB 435 cell variants were harvested with PBS/EDTA, and PE02JA cells were harvested with trypsin. Cells were incubated with 10 μg/ml mAb in Tris-buffered saline, 0.5% BSA (30 min on ice); washed; and stained with FITC anti-mouse IgG. To measure binding of the ligand mimetic antibody Fab WOW-1 (17), cells were incubated (30 min, 22 C°) with 10 μg/ml Fab in 137 mM NaCl, 2.7 mM KCl, 3.3 mM NaH_2PO_4, 3.8 mM Hepes, 1 mM $MgCl_2$, 400 μM $CaCl_2$, 5.5 mM glucose, and 1 mg/ml BSA, pH 7.4, with or without 250 μM $MnCl_2$ or 2 mM RGDS peptide. Cells were washed and incubated (30 min on ice) with Alexa-Fluor 488 anti-mouse IgG (BioSource International, Camarillo, CA) and analyzed on a Becton Dickinson FACScan.

Analytical Perfusion Studies. Breast cancer cell arrest during blood flow and interaction with platelets was measured as described (10). Briefly, tumor cells were suspended in human blood (anticoagulated with 50 nM H-D-Phe-Pro-Arg-chloro methyl ketone hydrochloride) and perfused over a collagen I matrix at a venous wall shear rate (50 s^{-1}, 4 dynes/cm^2). Adhesive events and cell interactions were visualized and recorded by fluorescence video or confocal laser microscopy (Zeiss) and quantified by image acquisition during perfusion at 50 predefined positions and computerized image analysis (METAMORPH; Universal Imaging, Media, PA). Tumor cells were stained with hydroethidine (Polysciences) (red fluorescence) (20 μg/ml, 30 min, 37°C), washed, and mixed with blood containing 10 μM mepacrine (green fluorescence). Blood cells, tumor cells, and platelets acquired green fluorescence and were visualized at 488/515 nm (excitation/emission). The tumor cells were identified by their unique red fluorescence at 543/590 nm. Integrin αvβ3- and αIIbβ3-dependent adhesive functions were tested with blocking anti-αvβ3 mAb VNR1 27.1 (18) or anti-αIIbβ3 mAb LJ CP8 (25) (80 μg/ml). Controls were nonfunction-blocking mAbs AV-8 (anti-αv) and AV-10 (anti-β3) (10).

Preparative Perfusion Studies to Isolate Platelet-Interactive Breast Cancer Cells. Platelet-interactive, arrest-competent variants of the parental MDA-MB 435 breast cancer cell line were isolated during sterile perfusion in human blood on collagen I at a wall shear rate of 50 s^{-1}. Unbound cells were removed by gentle washing with PBS. Thrombus formation was monitored by phase-contrast microscopy. The coverslips were cultured in complete Eagle's minimal essential medium as above, and decaying blood cells and platelets were removed by media changes. After 3 weeks, proliferating tumor cells from a given slide were pooled and resorted four more times to select breast cancer cells with the platelet-interactive phenotype. We generated five independently sorted MDA-MB 435 cell variants. Their abilities to undergo platelet-mediated arrest during blood flow were analyzed and quantified as above.

Haptotactic Migration Assay. Migration of the breast cancer cell variants toward purified extracellular matrix proteins was analyzed in transwells (8-μm pore size; Costar). Filter undersides (duplicates) were coated with 10 μg/ml human vitronectin, plasma fibronectin, 20 μg/ml fibrinogen, or BSA in PBS and blocked with 5% nonfat dry milk, 0.2% Tween 20 in PBS (2 h at 22°C). Cells were starved overnight in 0.5% FBS, harvested with PBS/EDTA, washed in migration buffer (Eagle's minimal essential medium), and seeded at 6×10^4 cells per upper transwell chamber. After 14 h at 37°C, 5% CO_2, filters were washed, and cells from the filter tops were removed, fixed, and stained (DiffQuick). Migrated cells were counted in 10 random optical fields per filter by two observers unaware of the conditions.

***In Vivo* Metastasis Assay.** To compare the metastatic potential of MDA-MB 435 breast cancer cell variants, 1×10^6 tumor cells were injected into the lateral tail vein of 6-week-old female C.B17/IcrTac *scid* mice (Taconic Farms) ($n = 8$). Forty-two days later, mice were killed, dissected, and analyzed by gross examination. The lungs were excised and fixed in Bouin's solution, and metastatic foci were counted at the lung surface under a dissecting microscope.

Results and Discussion

Metastatic Human Breast Cancer Cells Interact with Platelets and Arrest During Blood Flow. To test the hypothesis that tumor cell binding to platelets during blood flow is a critical property of metastatic tumor cells, we generated tumor- or metastasis-derived variants of the MDA-MB 435 human breast cancer cell line. Parental MDA-MB 435 cells were injected into the mammary fat pads of severe combined immunodeficient mice, and cell variants were retrieved from the resulting tumors or distant metastases to lymph nodes, lungs, bone, and the pleural cavity. These cell variants were compared for their ability to attach to activated platelets and undergo platelet-mediated arrest during blood perfusion *in vitro*. MDA-MB 435 parental cells largely failed to adhere or interact with platelets during blood flow. In contrast, cell variants derived from mammary fat pad tumors or distant metastases adhered and used platelet interaction for cell arrest (Fig. 1*A*). Importantly, primary metastatic cells isolated from a pleural effusion of a patient with advanced breast cancer exhibited a strong platelet-interactive phenotype and were incorporated into thrombi that formed at a collagen I matrix during blood perfusion. Tumor cells that bound to attached, activated platelets extended pseudopods and established shear-resistant contact with thrombi (Fig. 1*C*). Therefore, a platelet-interactive phenotype that promotes tumor cell arrest during blood flow correlated with a tumorigenic and metastatic phenotype in the tested human breast cancer cells.

Activated αvβ3 Supports Platelet-Dependent Breast Cancer Cell Arrest During Blood Flow. We reported that an interaction between melanoma cells and platelets during blood flow is mediated by tumor cell integrin αvβ3 and platelet integrin αIIbβ3 in the presence of connecting plasma proteins such as fibrinogen (10). To analyze whether platelet-supported arrest of tumor- or metastasis-derived human breast cancer cells depends on a similar mechanism, the cells were perfused in blood containing function-blocking anti-αvβ3 or anti-αIIbβ3 antibody. Arrest of mammary fat pad tumor- or metastasis-derived MDA-MB 435 cells and primary metastatic human breast cancer cells was strongly inhibited by anti-αvβ3 (Fig. 1*B*) and abolished by anti-platelet-αIIbβ3 (not shown). Similar results were obtained for all arrest-competent, platelet-interactive variants of the MDA-MB 435 cell model. Therefore, tumor cell integrin αvβ3 can mediate breast cancer cell arrest during blood flow through an interaction with platelets.

MDA-MB 435 parental cells failed to bind to platelets and arrest during blood flow, but the tumor- or metastasis-derived cell variants did bind in a platelet- and αvβ3-dependent manner. We tested whether this difference in binding patterns was caused by differences in αvβ3 expression levels. However, these differed

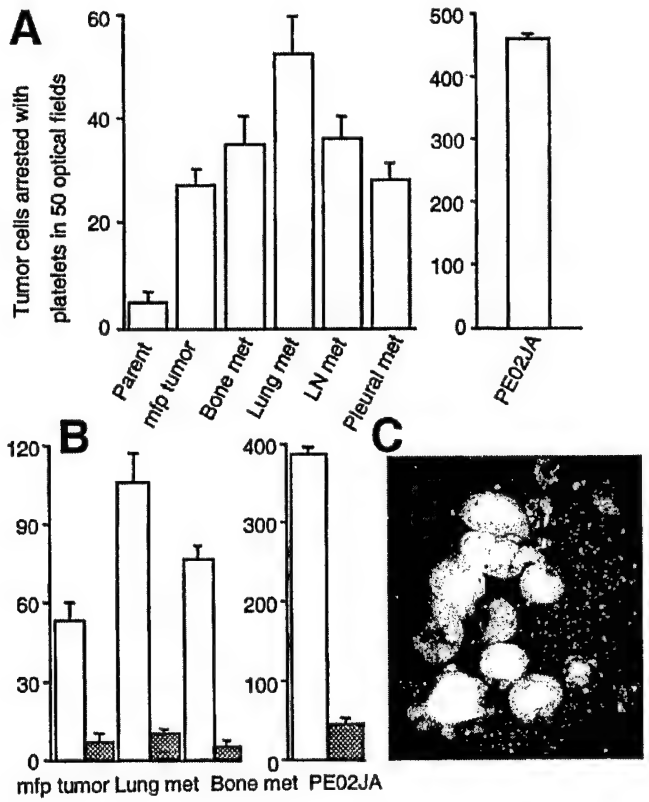

Fig. 1. Metastatic human breast cancer cells use $\alpha v\beta 3$ to interact with platelets and arrest during blood flow. (*A*) Primary metastatic human breast cancer cells (PE02JA) and MDA-MB 435 cell variants derived from mammary fat pad (mfp) tumors or metastases to bone, lungs, lymph node (LN), or the pleural cavity in mice, but not the parental MDA-MB 435 cell population at large, bind to activated platelets and thereby arrest during blood flow. Tumor cells stained with hydroethidine (red fluorescence) were suspended in human blood (containing mepacrine, green fluorescence) and perfused over a thrombogenic collagen I matrix at a venous wall shear rate (50 s^{-1}, 4 dynes/cm^2) (10). Platelets adhere to the matrix, become activated, and form thrombi, to which arrest-competent tumor cells attach (monitored by video microscopy and image acquisition at predefined positions with filter settings to discern platelet- and tumor cell-specific signals). None of the tumor cells attached directly to the matrix. Thrombus formation was not affected by tumor cell type. (*B*) Arrest-competent breast cancer cells use integrin $\alpha v\beta 3$ for attachment. Tumor cells in blood were perfused and analyzed as in *A* in the absence (open bars) or presence (hatched bars) of function-blocking anti-$\alpha v\beta 3$ mAb VNR1 27.1 (18). Columns represent the means of triplicate runs (±SD) with blood from the same donor. (*C*) Projection of confocal sections through a heteroaggregate of platelets and primary metastatic breast cancer cells, PE02JA (images acquired during perfusion). Attaching to a thrombus, tumor cells extend pseudopods for continued anchorage.

only slightly when comparing MDA-MB 435 parental cells and their tumor- or metastasis-derived variants (Table 1). Therefore, the distinct functional activities of $\alpha v\beta 3$ suggest that the integrin is present in a nonactivated state in the parental cell line, but in an activated state in the *in vivo* selected cell variants. The activation state can be defined by the platelet-interactive phenotype.

Parental MDA-MB 435 Human Breast Cancer Cells Contain a Subpopulation That Stably Expresses Activated $\alpha v\beta 3$. Our data are consistent with the idea that tumor cells expressing platelet-interactive $\alpha v\beta 3$ are present in the parental MDA-MB 435 cell line at a low frequency and that these were selected *in vivo* during tumor growth and metastasis. The MDA-MB 435 cell line is a polyclonal cell population, but its variants derived from distant metastases in mice are oligo- or monoclonal (26). We therefore asked whether cells expressing the platelet-interactive phenotype are present in the parental MDA-MB 435 parental cell population and can be isolated *in vitro* based on their ability to undergo platelet-mediated arrest during blood flow. To test this possibility, parental cells were suspended in normal donor blood and perfused over a thrombogenic collagen I matrix under sterile conditions. Attached cells were expanded and resorted four times to enrich cells with a platelet-interactive phenotype. Analytical perfusion experiments, in the absence or presence of function-blocking anti-$\alpha v\beta 3$ antibody, showed that all of five independently sorted variant cell populations expressed the platelet-interactive form of integrin $\alpha v\beta 3$. The extent of platelet interaction was similar to that observed in the *in vivo* selected metastatic cell variants (Fig. 2*A* shows two *in vitro* sorted cell populations, 05S05 and 10S05). The expression levels of integrin $\alpha v\beta 3$ were similar in the parental cell population and the *in vitro* selected variants (Fig. 2*B*). All *in vitro* isolated variants stably expressed the platelet-interactive phenotype over more than 15 passages in culture. This persistence of the phenotype confirms that the MDA-MB 435 parental cell line contains cells that express $\alpha v\beta 3$ in either of two activation states, a platelet-interactive or a noninteractive state. Unless under selective pressure, as during tumor growth or metastasis, the parental MDA-MB 435 cell population conserved the ratio of cells expressing the non-platelet-interactive versus the interactive form of $\alpha v\beta 3$. This conservation of this ratio was evident from repeated analytical blood perfusion experiments with parental MDA-MB 435 cells for more than 20 culture passages, during which the population at large maintained the non-platelet-interactive phenotype.

Integrin $\alpha v\beta 3$ Activation Results in the Platelet-Interactive, Arrest-Competent Phenotype in MDA-MB 435 Human Breast Cancer Cells. We established a correlation between the platelet-interactive and the metastatic phenotype of MDA-MB 435 breast cancer cells. We now sought to determine whether there is a causal link between these two phenomena. To test the hypothesis that the activated, platelet-interactive state of tumor cell integrin $\alpha v\beta 3$, but not the nonactivated state, promotes hematogenous metastasis, MDA-MB 435 cells were transfected with a $\beta 3$ mutant to express constitutively activated $\alpha v\beta 3$. To accomplish this transfection, a $\beta 3$-minus variant was selected from MDA-MB 435

Table 1. Integrin $\alpha v\beta 3$ expression in the human breast cancer cell model

	MDA-MB 435 parent	MDA-MB 435 mfp tumor	MDA-MB 435 bone met	MDA-MB 435 lung met	MDA-MB 435 lymph node met	MDA-MB 435 pleural met	PE02JA
$\alpha v\beta 3$	36.87	40.16	47.23	40.24	47.24	41.35	55.53
$\alpha 2\beta 1$	28.42	26.37	22.63	28.54	20.34	28.70	235.37

Variants of MDA-MB 435 cells were generated by injecting the parental cell line into the mammary fat pad (mfp) of severe combined immunodeficient mice and culturing their descendants from developing tumors or distant metastases to bone, lungs, lymph node, or the pleural cavity. PE02JA cells are primary metastatic breast cancer cells from a pleural effusion of a patient with advanced breast cancer. Integrin expression levels were determined by flow cytometry with anti-$\alpha v\beta 3$ mAb LM609 or anti-$\alpha 2$ mAb 12F1 and FITC-anti-mouse IgG. Values are median fluorescence intensities.

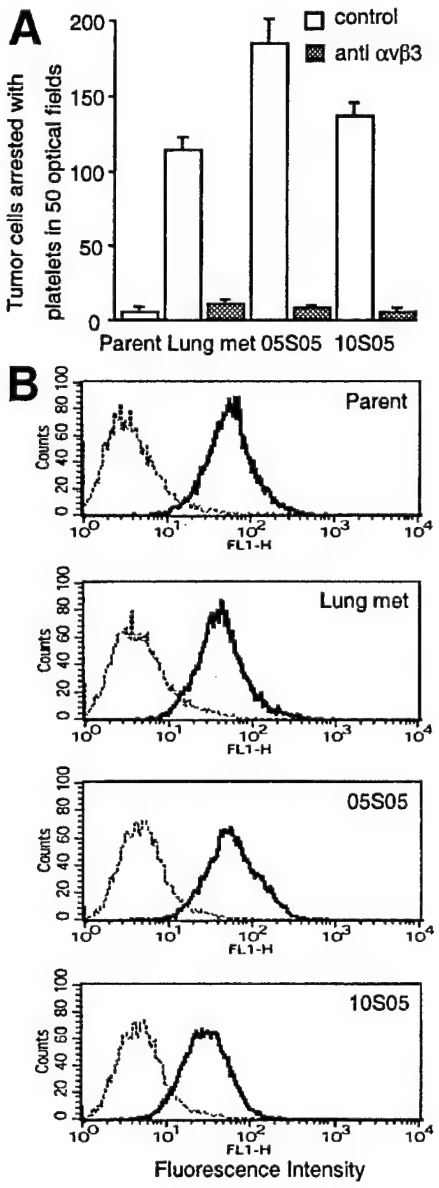

Fig. 2. MDA-MB 435 breast cancer cells contain an arrest-competent subset that expresses activated $\alpha v\beta 3$. Parental MDA-MB 435 breast cancer cells were suspended in human blood and perfused as in Fig. 1, but under sterile conditions. Arrested cells were expanded and resorted four times. (*A*) Two independently sorted polyclonal populations (05S05 and 10S05) analyzed for their ability to undergo platelet mediated arrest during blood flow (as in Fig. 1) in the absence (open bars) or presence (hatched bars) of function-blocking anti-integrin $\alpha v\beta 3$ mAb VNR1 27.1. Columns represent means of triplicate runs (±SD) with blood from the same donor. (*B*) Parental MDA-MB 435 cells (Parent) and their *in vivo* (Lung met) or *in vitro* (05S05 and 10S05) selected variants express integrin $\alpha v\beta 3$ at similar levels. Flow cytometric analysis was carried out on cells stained with mAb LM609 (anti-$\alpha v\beta 3$) (——) or isotype control (·····) and FITC-anti-mouse IgG.

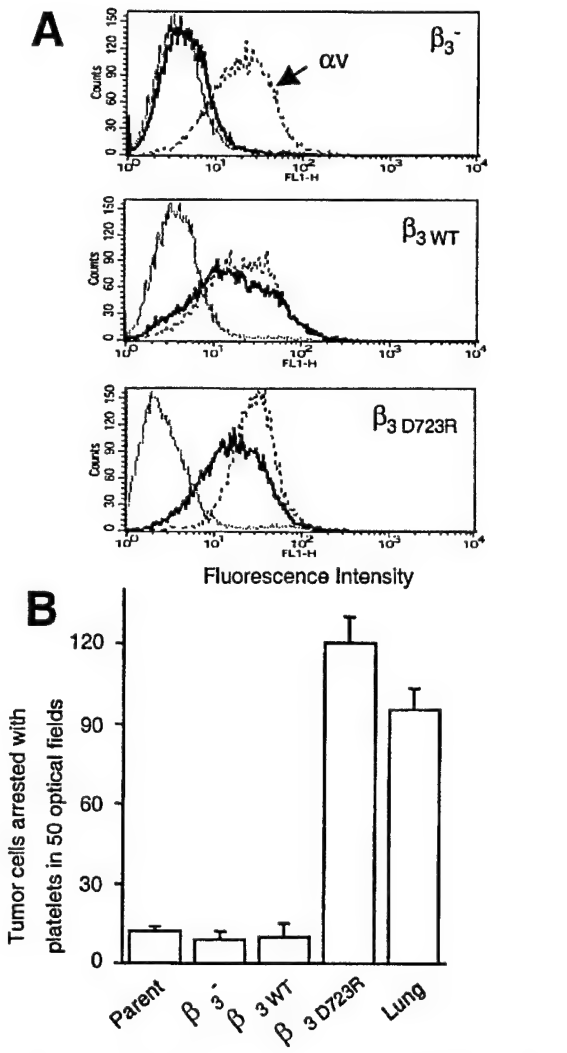

Fig. 3. Integrin $\alpha v\beta 3$ activation renders breast cancer cells platelet-interactive and arrest-competent. A variant lacking $\beta 3$ integrin expression ($\beta 3^-$) was selected from parental MDA-MB 435 cells by exposure to an anti-$\beta 3$ saporin conjugate. $\beta 3^-$ cells were stably transfected with the $\beta 3$ wild-type gene ($\beta 3_{WT}$) or constitutively activated mutant $\beta 3_{D723R}$. (*A*) Flow cytometric analysis of $\alpha v\beta 3$ expression. Cells stained with anti-αv (mAb AV-8, – – –) (10), anti-$\alpha v\beta 3$ (mAb LM609, ——), or isotype control (·····) and FITC-anti-mouse IgG. (*B*) Cells expressing constitutively activated $\alpha v\beta 3_{D723R}$, but not $\alpha v\beta 3_{WT}$, are platelet-interactive and arrest-competent. MDA-MB 435 parental cells (Parent), the $\beta 3$-lacking variant ($\beta 3^-$), transfectants ($\beta 3_{WT}$, $\beta 3_{D723R}$), or the *in vivo* selected metastatic variant (Lung) were perfused in human blood, and cell arrest was analyzed as in Fig. 1. Columns represent means of triplicate runs (±SD) with blood from the same donor.

parental cells by exposing the cells to a saporin-anti-$\beta 3$ antibody conjugate that selectively killed $\beta 3$-expressing cells (23). After five rounds of selection, a $\beta 3$-minus population was obtained that maintained this phenotype over multiple culture passages (Fig. 3*A*). These cells were transfected stably with cDNA encoding full-length human $\beta 3$ wild type, $\beta 3_{WT}$, or mutant $\beta 3_{D723R}$. Expression of the $\beta 3_{D723R}$ mutant results in constitutively activated platelet integrin $\alpha IIb\beta 3$ (24). It dimerizes with the αv subunit, and this dimerization results in an altered functional state of integrin $\alpha v\beta 3$ (27). Here, stable transfectants of MDA-MB 435 $\beta 3$-minus cells were generated that expressed either $\alpha v\beta 3_{WT}$ or mutant $\alpha v\beta 3_{D723R}$ at levels comparable to that of $\alpha v\beta 3$ in the parental cell line (Fig. 3*A*). The transfectants were analyzed *in vitro* for their ability to arrest during blood flow. Cells expressing mutant $\alpha v\beta 3_{D723R}$, but not those expressing $\alpha v\beta 3_{WT}$ or expressing no $\beta 3$, were able to arrest in a platelet-dependent manner similar to that of the *in vivo* selected metastatic MDA-MB 435 cell variants (Fig. 3*B*). $\alpha v\beta 3$ is the only $\beta 3$ integrin of MDA-MB 435 cells. Therefore, the expression of mutant $\beta 3_{D723R}$ resulted in functionally activated $\alpha v\beta 3$ that supported tumor cell arrest during blood flow through interaction with platelets.

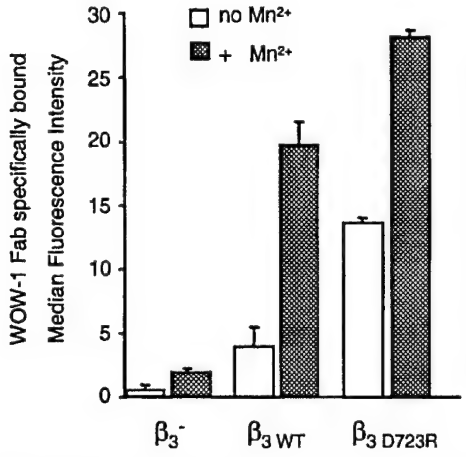

Fig. 4. Binding of the ligand-mimetic antibody Fab WOW-1 to functional variants of MDA-MB 435 breast cancer cells. Flow cytometric analysis of WOW-1 binding (activation-dependent anti-αvβ3 Fab) to variants of MDA-MB 435 breast cancer cells lacking β3 (β_3^-) or transfected with the β3 wild-type gene ($\beta3_{WT}$) or constitutively activated mutant $\beta3_{D723R}$. Cells were incubated with 10 μg/ml WOW-1 in the absence (open bars) or presence (hatched bars) of 250 μM $MnCl_2$, added to activate αvβ3, and stained with Alexa Fluor 488-anti mouse IgG. Shown is specific WOW-1 binding defined as that inhibited by 2 mM RGDS peptide (means of duplicate analyses ± SD).

Integrin αvβ3 Activation Promotes Binding of a Ligand-Mimetic Antibody and Enhances Breast Cancer Cell Migration Toward Vitronectin. The ability of integrin αvβ3 to support breast cancer cell arrest during blood flow in one functional state, but not the other, indicates strongly that αvβ3 exists in an activated and a nonactivated or less activated state in these tumor cells. To test whether the arrest-competent state of breast cancer cell integrin αvβ3 supports other cell functions differently than the non-arrest-competent state, we analyzed binding of the ligand-mimetic antibody WOW-1. WOW-1 is a genetically engineered Fab fragment that contains a RGD sequence in the context of the adenovirus penton base protein and serves as a monovalent ligand for αv integrins (17). Importantly, WOW-1 was generated on the framework of the PAC-1 Fab, which recognizes platelet integrin αIIbβ3 in an activation-dependent manner (28). Therefore, WOW-1 specifically reports an activated state of integrin αvβ3 (17). Here we show that MDA-MB 435 breast cancer cells expressing arrest-competent $\alpha v\beta3_{D723R}$ bound twice as much WOW-1 as the variant expressing non-arrest-competent $\alpha v\beta3_{WT}$ (Fig. 4). The two cell variants expressed αvβ3 at equivalent levels (Fig. 3). In the presence of Mn^{2+}, WOW-1 binding increased 2-fold in $\alpha v\beta3_{D723R}$-expressing cells but 5-fold in $\alpha v\beta3_{WT}$-expressing cells. This difference in the increase in WOW-1 binding indicates that $\alpha v\beta3_{D723R}$ already exists in a state of increased activation in the absence of exogenous agonist. Similar results were obtained by comparing MDA-MB 435 parental cells and the *in vivo* selected metastatic variant from the lung (not shown).

Integrin αvβ3-mediated cell migration on certain ligands is affected by the functional state of the receptor (29). To confirm the activated state of αvβ3 in the arrest-competent variants of our MDA-MB 435 breast cancer cell model, we analyzed cell migration toward matrix proteins. We tested vitronectin, fibronectin, and fibrinogen, which are ligands of αvβ3 and support cell adhesion through this receptor (16, 30). Integrin αvβ3 activation enhanced breast cancer cell migration toward vitronectin. Metastatic MDA-MB 435 cells from the lung and $\beta3_{D723R}$

Table 2. Haptotactic migration of functional variants of MDA-MB 435 breast cancer cells

	Lung	Parent	$\beta3_{D723R}$	$\beta3_{WT}$	$\beta3^-$
VN	473 ± 32	225 ± 18	1125 ± 85	478 ± 42	198 ± 15
FN	372 ± 42	441 ± 42	1085 ± 95	1205 ± 110	275 ± 22
Fg	33 ± 12	15 ± 11	70 ± 12	18 ± 6	6 ± 4

Migration toward vitronectin (VN), fibronectin (FN), or fibrinogen (Fg) was analyzed for MDA-MB 435 parental cells (parent), an *in vivo* selected metastatic variant (Lung), or the β3-integrin lacking variant (β_3^-) and its transfectants expressing either β3 wild-type ($\beta3_{WT}$) or constitutively activated $\beta3_{D723R}$. Cells were starved overnight in 0.5% FBS, and migration was assayed in transwell chambers (6×10^4 per well in duplicate, 14 h). Migrated cells were counted at the filter underside in 10 random optical fields per filter by two observers. Columns represent mean numbers of migrated cells per field (± SD). Data comparing the metastatic cell variant (Lung) to the parental cells and data comparing β3-lacking cells to its β3 transfectants are from independent experiments. Absolute numbers of migrated cells varied between experiments, but the ratios of migratory activities between the cell types remained constant.

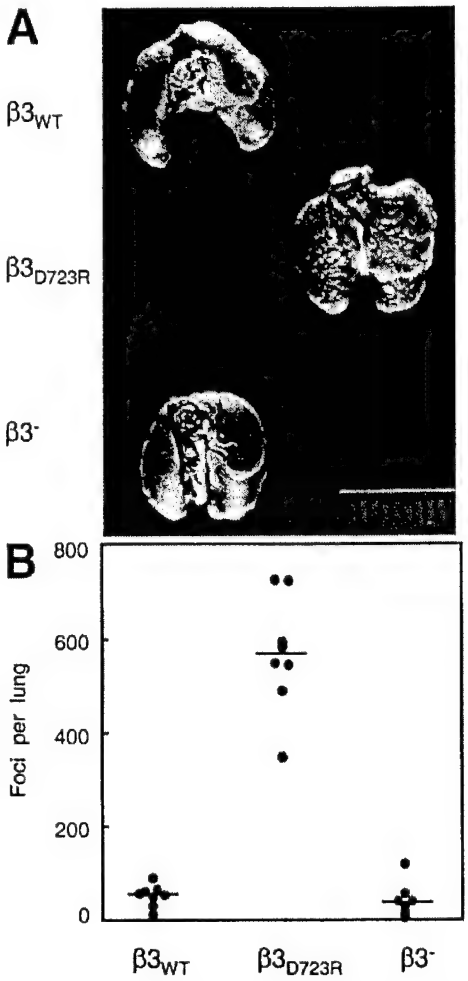

Fig. 5. Integrin αvβ3 activation controls metastatic potential in the MDA-MB 435 breast carcinoma cell model. (*A*) Lungs of female C.B17/IcrTac *scid* mice 42 days after i.v. injection of 1×10^6 tumor cells. The β3 integrin lacking cell variant and its transfectants expressing either $\alpha v\beta3_{WT}$ or $\alpha v\beta3_{D723R}$ were compared. The $\beta3_{D723R}$-expressing variant had the platelet-interactive phenotype and showed increased metastatic activity. (*B*) Number of metastatic foci at the lung surface. Data points are numbers of lung surface metastases for each animal; horizontal lines are median numbers of metastases per group ($n = 8$). Cells expressing activated $\alpha v\beta3_{D723R}$ produced a significantly larger number of metastases than cells lacking β3 or expressing nonactivated $\alpha v\beta3_{WT}$ ($P < 0.0001$ by the Kruskal–Wallis test).

transfectants (activated $\alpha v\beta 3$) migrated more actively toward vitronectin than the parental cells or $\beta 3_{WT}$ transfectants (nonactivated $\alpha v\beta 3$) (Table 2). Migration toward fibronectin was also mediated by $\alpha v\beta 3$, but was not affected by the receptor activation state. Low levels of vitronectin- and fibronectin-directed migration seen in the $\beta 3$-minus variant were likely supported by integrin $\alpha v\beta 5$ and $\alpha 5\beta 1$, respectively (both receptors were expressed by all MDA-MB 435 cell variants). The tested cell variants migrated poorly toward fibrinogen.

Together, the activated state of integrin $\alpha v\beta 3$ in breast cancer cells, defined here by the platelet-interactive, arrest-competent phenotype, was confirmed by increased binding of a ligand-mimetic antibody and increased support of cell migration toward vitronectin.

Integrin $\alpha v\beta 3$ Activation Controls the Metastatic Potential in MDA-MB 435 Breast Cancer Cells. To test whether activation of tumor cell integrin $\alpha v\beta 3$ affects the metastatic activity of breast cancer cells, MDA-MB 435 transfectants expressing either nonactivated $\alpha v\beta 3_{WT}$ or constitutively activated mutant $\alpha v\beta 3_{D723R}$ were injected into the circulation of severe combined immunodeficient mice. The ability of the cells to colonize the lungs was compared with that of the $\beta 3$-lacking cell variant. Metastatic activity was significantly enhanced ($P < 0.0001$) in cells expressing mutant $\alpha v\beta 3_{D723R}$ compared with cells expressing $\alpha v\beta 3_{WT}$ or no $\beta 3$ (Fig. 5). There was no difference between the latter two groups. Thus, in the MDA-MB 435 breast cancer cell model, expression of activated $\alpha v\beta 3$ resulted in a platelet-interactive phenotype and strongly increased metastatic activity.

It is currently unknown whether an interaction between breast cancer cells and platelets within the host circulation critically affects metastatic activity. The interaction of the tumor cells with platelets during blood flow *in vitro* allowed us to identify a functionally activated state of tumor cell integrin $\alpha v\beta 3$ that may promote metastasis through a combination of altered adhesive, migratory, and other cell functions. The platelet-interactive variants of the MDA-MB 435 cell model, identified by perfusion in human blood, also interacted with murine platelets when perfused in mouse blood (not shown). It is therefore possible that this mechanism promoted metastatic activity of the human breast cancer cells in the mouse model.

We showed that $\alpha v\beta 3$ can exist in breast cancer cells in distinct functional states. The activated but not the nonactivated state supported tumor cell arrest during blood flow through interaction with platelets. We established a correlation between the expression of activated $\alpha v\beta 3$ and the metastatic phenotype in the MDA-MB 435 human breast cancer cell model and in primary metastatic cells from a breast cancer patient. Importantly, we documented a causal relationship between the expression of activated $\alpha v\beta 3$ and the metastatic potential in MDA-MB 435 breast cancer cells, because expression of constitutively activated mutant $\alpha v\beta 3_{D723R}$, but not $\alpha v\beta 3_{WT}$, resulted in a significant increase in metastatic activity. These results demonstrate that human breast cancer cells can exhibit a platelet-interactive and metastatic phenotype that is controlled by the activation state of tumor cell integrin $\alpha v\beta 3$. This conclusion is consistent with a "two hit hypothesis" (31) in which $\alpha v\beta 3$ expression is necessary but not sufficient for successful breast cancer metastasis. Rather, additional as yet undefined factor(s) that control(s) the activation state of the integrin are required for metastatic dissemination. Consequently, alterations within tumors that lead to the aberrant control of integrin activation are expected to adversely affect the course of human breast cancer.

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Lectures

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Tumor cell-platelet interaction in metastatic disease

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Metastasis of solid tumors to major target organs often involves tumor cell dissemination via the blood stream. Clinical and experimental evidence suggests that platelets may play a role in this process. Cancer is often associated with a high incidence of thrombosis [1]. The most severe forms are disseminated intravascular coagulation, migratory thrombophlebitis and pulmonary embolism. But even in the absence of clinically detected thromboembolic events, coagulation parameters are frequently elevated in cancer patients, and platelet turnover is generally enhanced. A combination of anticoagulant medication with established anti-cancer regimens resulted in improved therapeutic effects in a variety of malignancies.

Dissemination of metastasizing cells within the blood stream is thought to be supported by an interaction between tumor cells and platelets [2]. Perhaps the most convincing evidence is that experimental thrombocytopenia reduced hematogenous tumor metastasis in animal models. This effect was reversed upon platelet infusion [3]. Furthermore, inhibition of specific platelet adhesive functions by monoclonal antibodies, or synthetic and naturally occurring RGD-containing peptides reduced experimental metastasis significantly in the majority of studies. Metastasis was inhibited regardless of the tumor cell ability to induce platelet aggregation. Two mechanisms of platelet involvement in tumor metastasis can be distinguished: first, the induction of platelet activation and platelet aggregation by tumor cells or their released factors, which may be accompanied by passive entrapment of tumor cells into platelet aggregates; and second, a specific adhesive interaction between platelets and tumor cells. Both of these mechanisms are influenced by blood flow and flow dependent shear forces that are present in the vasculature. Under these conditions, an interaction between tumor cells and platelets may facilitate metastatic dissemination in at least three major ways: First, heteroaggregate formation could support tumor cell arrest within the blood stream, as a prerequisite for tumor cell extravasation; Second, association of tumor cells with platelets may reduce tumor cell exposure to mechanical stress and shear forces, which have been hypothesized to be a main cause of rapid cancer cell death in the vasculature: Third, tumor cells surrounded by platelets could be protected against immune defense mechanisms. In a platelet rich microenvironment, the tumor cells are exposed to platelet released factors that can promote tumor cell survival and proliferation, even at the site of arrest within blood vessels [4]. Platelet derived particles can further induce or intensify tumor cell invasive activity [5].

Comparative experimental data justify the concept that cancer cell attachment inside blood vessels is not merely due to passive entrapment of the tumor cells based on their diameter, but that it rather depends on active and specific interactions between tumor cells and the endothelium, or exposed sites of the subendothelial matrix. Most tumor cells express a variety of adhesion receptors, which may support their attachment to counter receptors or ligands at the endothelium, and to components of the subendothelial matrix. These include von Willebrand Factor, collagen, fibronectin, laminin, vitronectin and glycosaminoglycans. Specific adhesion of various tumor cell types to each of these matrix components has been demonstrated under static conditions. Adhesion receptors involved include members of the integrin family, as well as non-integrin receptors. Static conditions, however, are unlikely to occur in the circulation. Passive entrapment of tumor cells in narrow capillary vessels may temporarily interrupt blood flow and favor direct tumor cell attachment to the endothelium, or to exposed sites of the subendothelial matrix. However, shear rates can also reach maximal levels in capillary passages. Therefore,

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adhesion mechanisms that mediate tumor cell attachment to the vessel wall have to be designed such that an adhesive interaction can establish under flow conditions and continuously withstand shear stress. Tumor cell interaction with platelets may provide the tumor cells with specific mechanisms that physiologically mediate platelet anchorage during blood flow. Recent experimental evidence supports this concept. Adhesion receptors of the selectin and integrin families with their ligands, were reported to promote tumor cell-platelet interaction during blood flow and promote metastasis from the blood stream. Platelet P-selectin was shown to bind tumor cell sialyl Lewis$^{x/a}$ glycoprotein, and this mechanism promoted arrest of tumor cell-platelet emboli in the vasculature of target organs [6]. Tumor cell arrest was inhibited by heparin, based on its ability to interfere with P-selectin mediated platelet-tumor cell binding, rather than on heparins anticoagulant effect [7].

We recently identified a specific mechanism for tumor cell-platelet interaction during blood flow. This mechanism is based on binding of platelet integrin $\alpha IIb\beta 3$ to tumor cell integrin $\alpha v\beta 3$ via divalent crosslinking ligands [8]. This was documented for human melanoma cells and fibrinogen as a possible ligand bridge. The interaction of tumor cells and platelets lead to tumor cell arrest and depended on platelet adhesive functions. The same mechanism supported human breast cancer cell arrest during blood flow in an established breast cancer cell model, and in freshly isolated metastatic cells from peripheral blood samples and malignant effusions of stage IV breast cancer patients [9] (Fig. 1). Importantly, the platelet interactive phenotype in breast cancer cells was associated with the metastatic phenotype. Metastasis derived variants of the breast cancer cell line, but not the parental cells themselves, were able to utilize integrin $\alpha v\beta 3$ for platelet binding and for arrest during blood flow. The parental tumor cells and their metastatic variants expressed $\alpha v\beta 3$ at the same levels. Therefore, integrin $\alpha v\beta 3$ can exist in breast cancer cells in an activated and a non-activated functional state. The expression of activated, platelet interactive $\alpha v\beta 3$ is associated with the metastatic phenotype. This was confirmed by introducing a constitutively activated mutant $\alpha 3$ subunit into a $\alpha 3$-negative variant of the parental breast cancer cells, which resulted in a platelet interactive and highly metastatic tumor cell phenotype (Fig. 1). Thus, alterations within tumors that lead to the aberrant control of integrin activation are expected to adversely affect the course of human breast cancer.

In order to clarify definitively, whether platelets indeed play a critical role in tumor metastasis, we are now establishing a new mouse model that lacks circulating platelets, and that is immune deficient to accept human tumor cell grafts. This model is based on a defect in the p45 subunit of the hematopoietic transcription factor NF-E2, which is required for platelet maturation [10]. We established the NF-E2 mutant allele on a scid/scid genetic background, and this model will allow us to analyze the metastatic activity of human tumor cells in the absence or presence of circulating platelets.

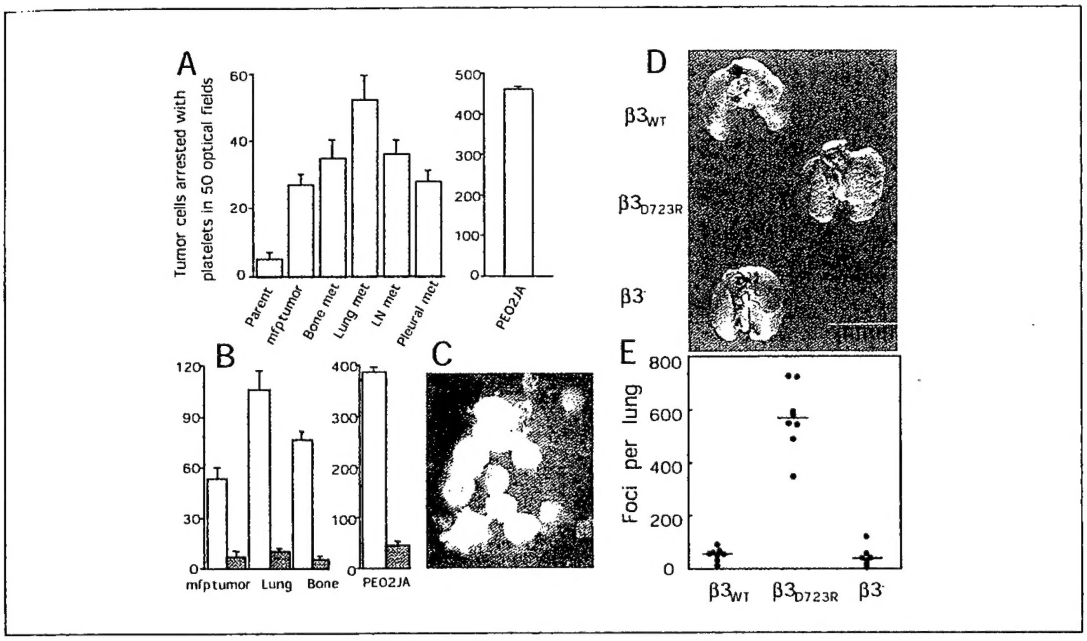

Figure 1. *Expression of activated integrin αvβ3 in human breast cancer cells promotes platelet-mediated tumor cell arrest during blood flow and experimental metastasis.*

A. *MDA-MB 435 cell variants derived from mammary fat pad (mfp) tumors or metastases (bone, lung, lymph node or the pleural cavity) in SCID mice, but not the parental cell population at large, bind to activated platelets and utilize this mechanism for cell arrest during blood flow. Tumor cells were stained with hydroethidine (red fluorescence), suspended in normal donor blood containing mepacrine (green fluorescence) and 50 nM H-D-Phe-Pro-Arg-chloromethyl ketone hydrochloride as anticoagulant. This suspension was perfused over a thrombogenic collagen I matrix at a venous wall shear rate of 50 sec^{-1} (4 dynes/cm^2) [8]. Under these conditions, platelets attach to the matrix, become activated and form thrombi. During perfusion, tumor cell interaction with thrombi was monitored by video microscopy and image acquisition at predefined positions with filter settings that discern platelet specific and tumor cell specific fluorescent signal. Thrombus formation at the matrix and a negligible number of directly attached tumor cells (not shown) were unaffected by the tumor cell type. The right panel shows primary metastatic tumor cells isolated from a pleural effusion of a patient with stage IV breast cancer (PE02JA) analyzed under the same conditions. The y-axes in A and B denote the number of tumor cells that arrested through association with platelets.* ***B.*** *Breast cancer cell interaction with platelets depends on tumor cell integrin αvβ3 and platelet integrin αIIbβ3 function. Mammary fat pad (mfp) tumor or metastasis (lung, bone) derived variants of MDA-MB 435 cells were analyzed as above in the absence (open bars) or presence (hatched bars) of 80 μg/ml function blocking anti αvβ3 mab VNR1 27.1. This antibody inhibits tumor cell platelet interaction, but not thrombus formation. Anti-αIIbβ3 mab LJ-CP8 inhibits thrombus formation and abolishes tumor cell binding (not shown). Note that all tested MDA-MB 435 cell variants express equivalent levels of αvβ3, but no platelet integrin αIIbβ3 (not shown).* ***C.*** *Projection of confocal sections through a breast cancer cell containing thrombus, acquired during blood perfusion. These tumor cells (PE02JA) were freshly isolated from a pleural effusion of a patient with advanced breast cancer and displayed a highly platelet interactive phenotype (right panel in A).* ***D.*** *Activation of integrin αvβ3 promotes metastasis in MDA-MB 435 human breast cancer cells. Comparison of in vitro generated variants of MDA-MB 435 cells that either lack α3 integrin expression (α3 minus) or were transfected to express αvβ3 wild type (α3$_{WT}$) or constitutively activated mutant avb3 $_{D723R}$ (α3$_{D723R}$). The cell variants (1x10^6) were injected into the lateral tail veins of 8-week old female C.B-17 scid/scid mice, and metastatic foci analyzed at the surface of the lungs after 42 days. Representative lungs are shown. E. Numbers of foci per lung (group size n=8). Cells expressing activated αvβ3$_{D723R}$ produced significantly more metastases than cells lacking α3 or expressing non-activated αvβ3 $_{WT}$ ($p < 0.0001$ by the Kruskal Wallis test).*

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